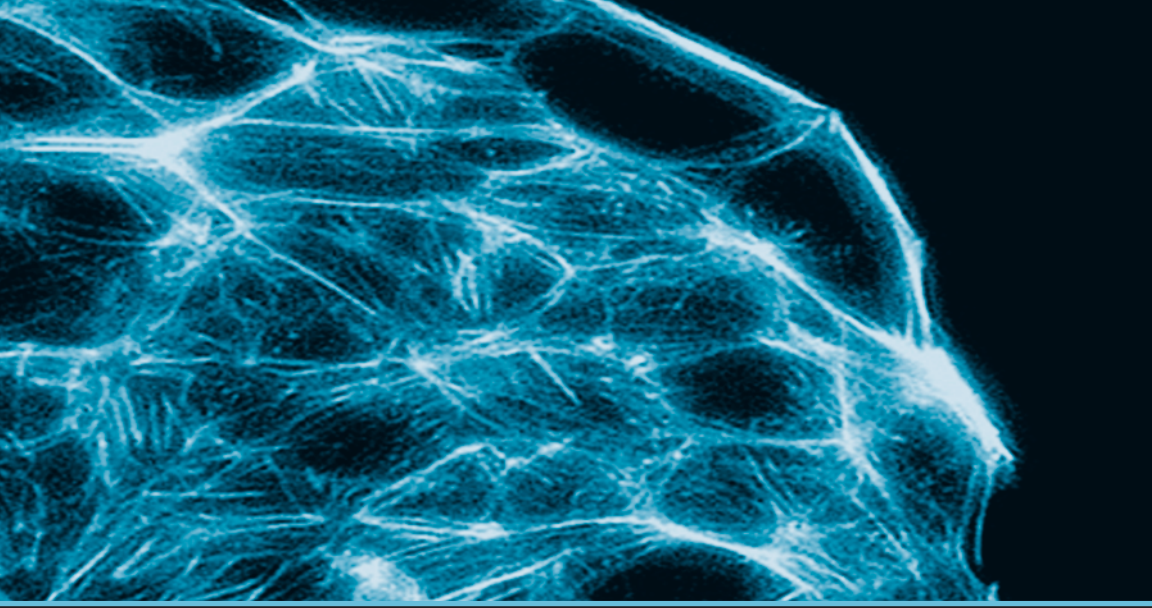

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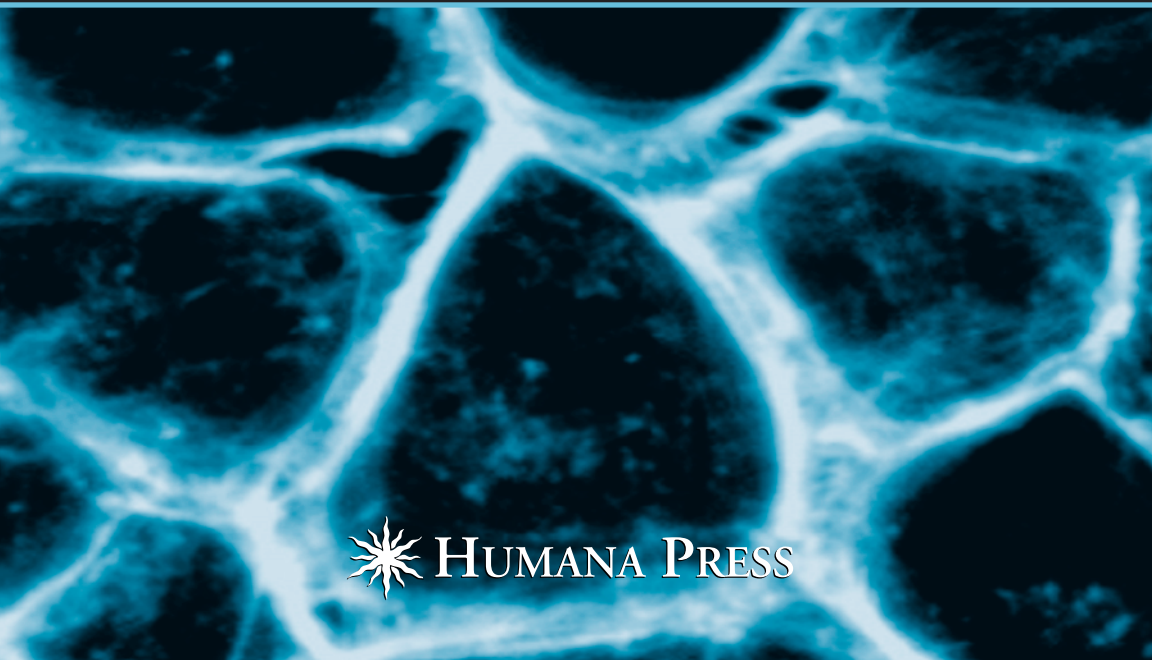

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Jian-Ying Wang

Departments of Surgery and Pathology, University of Maryland School of Medicine and Baltimore Veterans Affairs Medical Center, Baltimore, MD

Robert A. Casero, Jr.

The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, MD

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Preface

Polyamines are organic cations found in all eukaryotic cells and intimately involved in, and required for, distinct biological functions. An increasing body of evidence indicates that the regulation of cellular polyamines is a central convergence point for the multiple signaling pathways driving various cellular functions. Over the last decade, considerable progress has been made in understanding the molecular functions of cellular polyamines. These significant findings provide a fundamental basis to not only define the exact role of polyamines in physiology, but also to develop new therapeutic approaches for cancers and other diseases.

The major objective of this book is to provide a timely and long lasting guide for investigators in the fields of polyamines, physiology, pharmacology, and cancer research. It will provide a foundation based on research and address the potential for subsequent applications in clinical practice. *Polyamine Cell Signaling: Physiology, Pharmacology, and Cancer Research* is divided into four main parts:

Part I:	Polyamines in Signal Transduction of Cell Proliferation
Part II:	Polyamines in Cellular Signaling of Apoptosis, Carcinogenesis, and Cancer Therapy
Part III:	Polyamines in Cell Motility and Cell–Cell Interactions
Part IV:	Polyamine Homeostasis and Transport

This book not only covers the current state-of-the-art findings relevant to cellular and molecular functions of polyamines, but also provides the underlying conceptual basis and knowledge regarding potential therapeutic targeting of polyamines and polyamine metabolism. These points are addressed by internationally recognized experts in their contributions to this book.

We would like to take this opportunity to thank Humana Press, especially Mr. Harvey Kane and Ms. Erika Wasenda, who have made a great effort to make this book possible. We are indebted to all the contributors who have shared and contributed their invaluable research experiences and knowledge with us and to the medical community at large. And last but not least, we express our sincere thanks to our families for their generous support throughout the years.

Jian-Ying Wang
Robert A. Casero, Jr.

Contents

Contributors

LEENA ALHONEN • *A. I. Virtanen Institute for Molecular Sciences, University of Kuopio, Kuopio, Finland*

ALLAN A. ANCHETA • *Department of Biochemistry, University of California, Riverside, CA*

MARIE AUDETTE • *Oncological and Molecular Endocrinology Research Center and Department of Medical Biology, Laval University, Quebec, Canada*

NAVEEN BABBAR • *The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, MD*

CRAIG V. BYUS • *Department of Biochemistry, University of California, Riverside, CA*

ROBERT A. CASERO, JR. • *The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, MD*

RUPESH CHATURVEDI • *Department of Medicine, Division of Gastroenterology, Vanderbilt University School of Medicine, Nashville, TN*

JOHN L. CLEVELAND • *Department of Biochemistry, St. Jude Children's Research Hospital, Memphis, TN*

VINCENT CUIJPERS • *Department of Pathology, University Medical Center Nijmegen, Nijmegen, The Netherlands*

JENNIFER FLEISCHER • *The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, MD*

ALISON V. FRASER • *The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, MD*

BRUNO GAGNON • *Oncological and Molecular Endocrinology Research Center and Departments of Anatomy and Physiology, Laval University, Quebec, Canada*

EUGENE W. GERNER • *Department of Cell Biology and Anatomy, University of Arizona, Arizona Cancer Center, Tucson, AZ*

MARK N. GILLESPIE • *Department of Pharmacology, University of South Alabama College of Medicine, Mobile, AL*

SUSAN K. GILMOUR • *Lankenau Institute for Medical Research, Wynnewood, PA*

EMANUELE GIORDANO • *Department of Biochemistry "G. Moruzzi," University of Bologna, Bologna, Italy*

AMY HACKER • *The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, MD*

COLIN HANFREY • *Molecular Metabolism Group, Food Safety Science Division, Institute of Food Research, Norwich Research Park, Colney, Norwich, United Kingdom*
LEO HAWEL, III • *Division of Biomedical Sciences, University of California, Riverside, CA*
CHERYL A. HOBBS • *Lankenau Institute for Medical Research, Wynnewood, PA*
ALUN HUGHES • *Department of Medicine and Therapeutics, University of Aberdeen, College of Life Sciences and Medicine, Foresterhill, Aberdeen, Scotland*
MERVI T. HYVÖNEN • *A. I. Virtanen Institute for Molecular Sciences, University of Kuopio, Kuopio, Finland*
KAZUEI IGARASHI • *Graduate School of Pharmaceutical Sciences, Chiba University, Chiba, Japan*
JUHANI JÄNNE • *A. I. Virtanen Institute for Molecular Sciences, University of Kuopio, Kuopio, Finland*
AKI JÄRVINEN • *A. I. Virtanen Institute for Molecular Sciences, University of Kuopio, Kuopio, Finland*
LEONARD R. JOHNSON • *Department of Physiology, University of Tennessee, College of Medicine, Memphis, TN*
KEIKO KASHIWAGI • *Graduate School of Pharmaceutical Sciences, Chiba University, Chiba, Japan*
TUOMO A. KEINÄNEN • *A. I. Virtanen Institute for Molecular Sciences, University of Kuopio, Kuopio, Finland*
HARLEY T. KURATA • *Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, MO*
MARKKU LAAKSO • *A. I. Virtanen Institute for Molecular Sciences, University of Kuopio, Kuopio, Finland*
ANDREA MANNI • *Division of Endocrinology, Diabetes, and Metabolism, Department of Medicine, Pennsylvania State University College of Medicine, Hershey, PA*
TRACY MURRAY-STEWART • *The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, MD*
COLIN G. NICHOLS • *Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, MO*
JONAS A. NILSSON • *Department of Molecular Biology, Umea University, Umea, Sweden*
JACK W. OLSON • *Department of Pharmacology, University of South Alabama College of Medicine, Mobile, AL*
STINA M. OREDSSON • *Department of Cell and Organism Biology, Lund University, Lund, Sweden*

Lo Persson • *Department of Experimental Medical Science, Lund University, Lund, Sweden*
Marko Pietilä • *A. I. Virtanen Institute for Molecular Sciences, University of Kuopio, Kuopio, Finland*
Eija Pirinen • *A. I. Virtanen Institute for Molecular Sciences, University of Kuopio, Kuopio, Finland*
Richard Poulin • *Oncological and Molecular Endocrinology Research Center and Departments of Anatomy and Physiology, Laval University, Quebec, Canada*
Jaladanki N. Rao • *Department of Surgery, University of Maryland School of Medicine and Baltimore Veterans Affairs Medical Center, Baltimore, MD*
Ramesh M. Ray • *Department of Physiology, University of Tennessee, College of Medicine, Memphis, TN*
Serge Rivest • *Laboratory of Molecular Endocrinology, CHUL Research Center and Department of Anatomy and Physiology, Laval University, Quebec, Canada*
Johannes C. Romijn • *Department of Urology, Erasmus University, Rotterdam, The Netherlands*
Joseph Satriano • *Division of Nephrology–Hypertension, Department of Medicine and the Stein Institute for Research on Aging, University of California, San Diego, and Veterans Affairs San Diego Healthcare System, La Jolla, CA*
Raymond G. Schipper • *Department of Food Chemistry, Wageningen Center for Food Sciences, Wageningen, The Netherlands*
Lisa M. Shantz • *Department of Cellular and Molecular Physiology, Pennsylvania State University College of Medicine, Hershey, PA*
Denis Soulet • *Laboratory of Molecular Endocrinology, CHUL Research Center and Department of Anatomy and Physiology, Laval University, Quebec, Canada*
David E. Stringer • *Department of Cell Biology and Anatomy, University of Arizona School of Medicine and Arizona Cancer Center, Tucson, AZ*
Suvikki Suppola • *A. I. Virtanen Institute for Molecular Sciences, University of Kuopio, Kuopio, Finland*
Thresia Thomas • *Department of Environmental and Occupational Medicine, University of Medicine and Dentistry of New Jersey–Robert Wood Johnson Medical School, New Brunswick, NJ and The Cancer Institute of New Jersey, New Brunswick, NJ*
T. J. Thomas • *Department of Medicine, University of Medicine and Dentistry of New Jersey–Robert Wood Johnson Medical School, New Brunswick, NJ and The Cancer Institute of New Jersey, New Brunswick, NJ*

SRIPRIYA VENKITESWARAN • *Department of Medicine, University of Medicine and Dentistry of New Jersey–Robert Wood Johnson Medical School, New Brunswick, NJ and Department of Nutritional Sciences, Rutgers, The State University of New Jersey, New Brunswick, NJ*

ALBERT A. J. VERHOFSTAD • *Department of Pathology, University Medical Center Nijmegen, Nijmegen, The Netherlands*

AJIT K. VERMA • *Department of Human Oncology, University of Wisconsin School of Medicine, Madison, WI*

HEATHER M. WALLACE • *Department of Medicine and Therapeutics, University of Aberdeen, College of Life Sciences and Medicine, Foresterhill, Aberdeen, Scotland*

JIAN-YING WANG • *Departments of Surgery and Pathology, University of Maryland School of Medicine and Baltimore Veterans Affairs Medical Center, Baltimore, MD*

YANLIN WANG • *The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, MD*

KEITH T. WILSON • *Department of Medicine, Division of Gastroenterology, and Department of Cancer Biology, Vanderbilt University School of Medicine, Nashville, TN*

PATRICK M. WOSTER • *Department of Pharmaceutical Sciences, Wayne State University, Detroit, MI*

I

Polyamines in Signal Transduction of Cell Proliferation

1

Polyamine Structure and Synthetic Analogs

Patrick M. Woster

1. Introduction

The polyamines putrescine (1,4-diaminobutane), spermidine (1,8-diamino-4-aza-octane, 2), and spermine (1,12-diamino-4,9-diazadodecane, 3) (Fig. 1) are ubiquitous polycationic compounds that are found in significant amounts in nearly every prokaryotic and eukaryotic cell type. Spermidine and spermine primarily exist in aqueous solution at pH 7.4 as fully protonated polycations and possess the pKa values indicated in Fig. 1 *(1)*. This high degree of positive charge is an important factor in the biological functions of these molecules, and, as will be discussed later in this chapter, alterations in the pKa of polyamine nitrogens can affect and disrupt their cellular function. Polyamines are widely distributed in nature and are known to be required in micromolar to millimolar concentrations to support a wide variety of cellular functions. However, data that establish the precise role of the polyamines and their analogs in cellular processes are incomplete. The ongoing identification of new functions for the polyamines ensures that new avenues for research are arising continuously in an extremely diverse set of disciplines. The human and mammalian pathways for polyamine metabolism have been extensively studied, and analogous pathways have been elucidated for a relatively small number of organisms. There are important interspecies differences in polyamine metabolism, especially among eukaryotic cells, plants, and some bacteria and protozoa. In some prokaryotes, only putrescine and spermidine are synthesized, whereas in other cases, such as certain thermophilic bacteria, polyamines with chains longer than spermine are found. In some parasitic organisms, there are additional enzymes that are not present in the host cell, and, as such, provide a target for the design of specific antiparasitic agents. The enzymes involved in human and mammalian polyamine metabolism are reasonably similar, and inhibitors targeted to these enzymes rely on the observation that polyamine metabolism is accelerated, and polyamines are required in higher quantities, in target cell types. The diversity of biological research in the polyamine field is the subject of an excellent book *(2)*. Keeping in mind the diverse nature of polyamine distribution and function, it is reasonable

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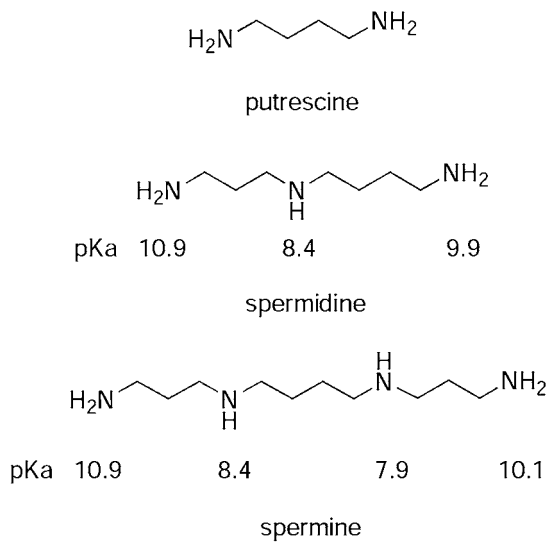

Fig. 1. Structures of putrescine, spermidine, and spermine, and pKa values for spermidine and spermine.

to assume that carefully designed polyamine analogs could have the potential to disrupt polyamine metabolism, and thus such agents have been investigated as potential therapeutic agents in vitro and in vivo. The polyamine pathway represents an important target for chemotherapeutic intervention because depletion of polyamines results in the disruption of a variety of cellular functions and may, in specific cases, result in cytotoxicity *(3,4)*. This chapter will summarize the development of synthetic derivatives of the polyamines, and describe their use as potential chemotherapeutic agents. A comprehensive review of polyamine biosynthesis inhibitors *(4)* and a review of the role of polyamines in normal and tumor cell metabolism *(5)* have recently been published.

2. Polyamine Biochemistry

The biosynthesis and catabolism of the polyamines putrescine, spermidine, and spermine are carefully controlled processes in all eukaryotic cell types. The mammalian polyamine biosynthetic pathway is shown in Fig. 2. Although definitive mechanisms for the various functions of the polyamines have not been fully elucidated, it is known that they are absolutely required for normal cell homeostasis. Inhibition of the polyamine pathway, therefore, is viewed as a valid target for the design of antitumor or antiparasitic agents. Available compounds that specifically inhibit individual enzymes in the pathway are extremely useful as research tools to elucidate the cellular functions of the naturally occurring polyamines. Specific inhibitors have now been developed for the enzymes in the forward polyamine biosynthetic pathway, ornithine decarboxylase (ODC), *S*-adenosylmethionine decarboxylase (AdoMet-DC), and for the aminopropyl-

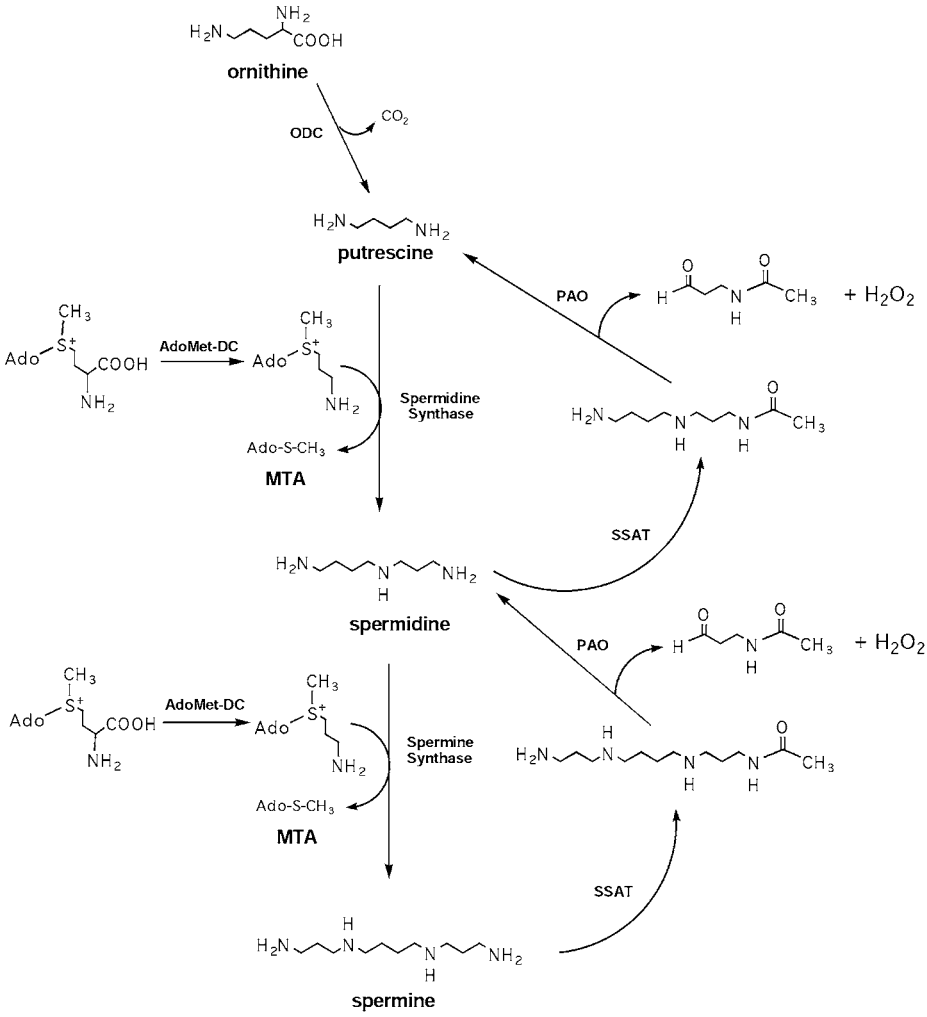

Fig. 2. The mammalian polyamine metabolic pathway.

transferases spermidine synthase and spermine synthase. These inhibitors produce a variety of responses ranging from cessation of cell growth to overt cytotoxicity *(6,7)*. The range of these activities appears to be both agent- and cell type-specific.

Polyamine metabolism can be viewed as having forward and reverse component pathways, although careful cellular control of these enzymes and the polyamine transporter act in concert to maintain appropriate levels of the individual polyamines. Ornithine is converted to putrescine by ODC, a typical pyridoxal phosphate-requiring amino acid decarboxylase. ODC is one of the control points in the pathway, producing a product that is committed to polyamine biosynthesis. ODC levels are modulated by

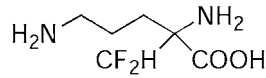

Fig. 3. The structure of α-difluoromethylornithine (DFMO).

synthesis and degradation of ODC protein, with a half-life of about 10 min. In mammalian cells, the degradation of ODC is facilitated by a specific ODC-antizyme *(8)*, a protein that also appears to downregulate polyamine transport. Competitive inhibitors of ODC have proven to be of limited value, and most useful inhibitor of ODC to date, α-difluoromethylornithine (DFMO;Fig. 3) *(9)*, is an irreversible inactivator of the enzyme. The discovery of DFMO provided an enormous stimulus to the field of mammalian polyamine biology, and led to marketing of the drug as a treatment for *Pneumocystis carinii* secondary infections. DFMO is also quite effective for the treatment of late-stage West African trypanosomiasis.

Putrescine is next converted to spermidine by the aminopropyltransferase spermidine synthase. A second closely related but distinct aminopropyltransferase, spermine synthase, then adds an additional aminopropyl group to spermidine to yield spermine, the longest mammalian polyamine. The byproduct for the spermidine and spermine synthase reactions is 5′-methylthioadenosine, generated from the cosubstrate, decarboxylated *S*-adenosylmethionine (dc-AdoMet). 5′-Methylthioadenosine is a potent product inhibitor for the aminopropyl transfer process, and must be rapidly hydrolyzed by 5′-methylthioadenosine-phosphorylase to maintain the forward pathway. Selective inhibition of the individual aminopropyltransferases has proven to be a significant problem because of the similarity of the reactions catalyzed by the two enzymes. The transition state analogs *S*-adenosyl-1,8-diamino-3-thiooctane *(10)* and *S*-adenosyl-1,12-diamino-3-thio-9-azadodecane *(11)* remain the only known specific inhibitors of the individual aminopropyltransferases.

The aminopropyl donor for both aminopropyltransferases is dc-AdoMet, produced from AdoMet by the action of AdoMet-DC. AdoMet-DC, like ODC, is a highly regulated enzyme in mammalian cells and belongs to a small class of proteins known as pyruvoyl enzymes. All of the known forms of AdoMet-DC contain a covalently bound pyruvate prosthetic group that is required for activity *(12)*, and formation of a Schiff's base between AdoMet and this pyruvate is a prerequisite for the reaction to occur. The antileukemic agent methylglyoxal *bis*(guanylhydrazone) (Fig. 4) is a potent competitive inhibitor of the putrescine-activated mammalian enzyme, with a K_i value of less than 1 μ*M* *(6)*, but is of limited use as a chemotherapeutic agent because of excessive toxicity. The AdoMet analog 5′-{[(*Z*)-4-amino-2-butenyl]methylamino}-5′-deoxyadenosine (AbeAdo;Fig. 4), is a potent enzyme-activated inhibitor of AdoMet-DC from *Escherichia coli*, and produces a long-lasting, dose-dependent decrease in AdoMet-DC activity in vivo *(13)*. Several additional inactivators of AdoMet-DC have been described *(4)*.

The so-called reverse polyamine metabolic pathway provides further control of cellular polyamine levels through acetylation and subsequent oxidative deamination processes. In the cell nucleus, spermidine is acetylated on the four carbon end by sper-

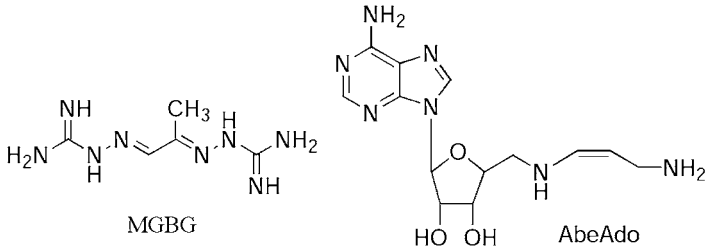

Fig. 4. Structures of the AdoMet-DC inhibitors of MGBG and AbeAdo.

midine-N^8-acetyltransferase, possibly altering the compound's binding affinity for DNA. A specific deacetylase can then reverse this enzymatic acetylation *(14)*. Neither of these enzymes affects the level of histone acetylation. Cytoplasmic spermidine and spermine are acetylated on the three carbon end by spermidine/spermine-N^1-acetyltransferase (SSAT;Fig. 2) *(4,15)*. This enzyme is the first and rate-limiting step in the catabolic interconversion of putrescine, spermidine, and spermine; its kinetics and substrate specificity have been described elsewhere *(4)*. Acetylated spermidine or spermine can be exported from the cell or oxidized by acetylpolyamine oxidase (PAO) to form 3-acetamidopropionaldehyde and either putrescine or spermidine, respectively (Fig. 2). SSAT and PAO together serve to reverse polyamine biosynthesis, facilitating the interconversion of cellular polyamines. It is important to note that the combination of the highly regulated catabolic enzyme SSAT, coupled with the finely controlled synthetic enzymes ODC and AdoMetDC, allow the cell considerable control of intracellular polyamine concentrations.

A final method of controlling intracellular polyamine levels is afforded by one or more specific polyamine transport mechanisms *(16,17)*. To date, the polyamine transport system in *E. coli* has been the most completely studied, resulting in the isolation of a transporter gene and a series of protein gene products designated PotA–PotF *(18)*. Specific polyamine transporters have been detected in yeast, *Trypanosoma cruzi* epimastigotes, *Crithidia fasciculata*, and *Leishmania donovani*. The process of polyamine transport in mammalian cells is poorly understood, and, to date, none of the proteins involved has been isolated and sequenced. Numerous groups are working to elucidate the mechanism(s) of transport, and the effects of regulation of transport, in normal and tumor cell lines. Several factors have been shown to alter the polyamine transport system, and, as a result, cellular homeostasis. It is worth noting that the polyamine system is significantly upregulated in a variety of tumor cells; thus, this system is regarded as a potential target for cancer chemotherapy *(19)*. Efforts to synthesize specific polyamine transport inhibitors have recently begun in several laboratories; a complete discussion of these efforts is beyond the scope of this chapter and has been previously reviewed *(17,19)*.

2.1. Symmetrical, Terminally Alkylated Polyamine Analogs

The development of analogs of spermidine and spermine as potential antitumor agents was initiated in the mid-1980s. Initially, these analogs were structurally similar

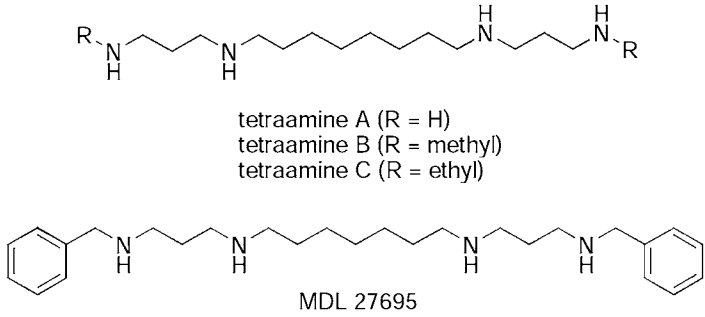

Fig. 5. The earliest known polyamine analogs with antitumor effects.

to the natural polyamines in that they had terminal primary amine groups, with variations in the length of the intermediate carbon chains. Edwards and coworkers synthesized a series of diamines and triamines related to spermidine and a series of tetraamines derived from 1,8-diaminooctane; these analogs were evaluated for antitumor activity in cultured L1210 cells *(20)*. In the series of diamines and triamines, substitution of alkyl groups at the terminal nitrogens, or replacing the central nitrogens with other heteroatoms, failed to produce spermidine analogs with antitumor effects superior to norspermidine. However, compounds with eight carbons between the central nitrogens, such as tetraamine A (Fig. 5), generally showed significant antitumor activity. Tetraamine A increased survival time in male mice inoculated with L1210 leukemia from 7.7 to 16.2 d. Coadministration of spermidine was shown to reverse the antitumor activity of this compound, presumably because of a competition for the polyamine transport system, whereas coadministration of a polyamine oxidase inhibitor potentiated the observed antitumor activity, suggesting that these analogs may be metabolized by polyamine oxidase. Tetraamines B and C (R = CH_3 or R = CH_2CH_3, respectively) were also active in the L1210 model, but substitution of larger alkyl groups resulted in a reduction of activity. In a related study, a series of *bis*benzyl analogs related to MDL 27695 and an additional series of substituted tetraamines were evaluated for the ability to inhibit proliferation of HeLa cells *(21)*. The *bis*(benzyl)polyamine analog MDL 27695 (Fig. 5) and tetraamine A were active antiproliferative compounds, exhibiting IC_{50} values of 5 and 50 μ*M*, respectively. Interestingly, no correlation between the DNA binding properties and antitumor activity of these analogs was detected.

Subsequent attempts to develop polyamine analogs as potential modulators of polyamine function focused on the synthesis of symmetrical, terminally substituted *bis*(alkyl)polyamines. These analogs were designed in response to the finding that natural polyamines use several feedback mechanisms that autoregulate their synthesis *(22)* and that they can be taken into cells by the energy-dependent transport systems described previously. Several symmetrically substituted polyamine analogs have been synthesized that enter the cell using the polyamine transport system. These analogs specifically slow the synthesis of polyamines by downregulation of the biosynthetic

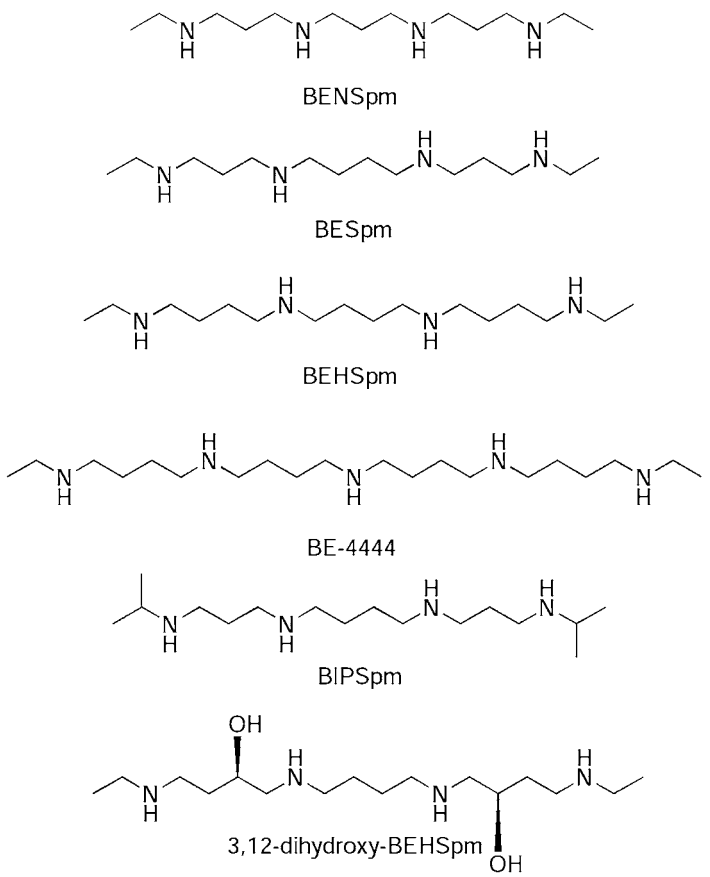

Fig. 6. Structures of *bis*(ethyl)polyamine analogs BENSpm, BESpm, BEHSpm, BIPSpm, BE-4444, and 3,12-dihydroxy-BEHSpm.

enzymes ODC and AdoMet-DC, but cannot substitute for the natural polyamines in terms of their cell growth and survival functions *(4,23)*. As will be discussed, some but not all alkylpolyamine analogs are potent inducers of SSAT in cultured tumor cells, an effect that leads to the induction of apoptosis. The most successful of the symmetrically substituted polyamine analogs to date are the *N,N′-bis*(ethyl)polyamines shown in Fig. 6: *bis*(ethyl)norspermine (BENSpm), *bis*(ethyl)spermine (BESpm), *bis*(ethyl)homospermine (BEHSpm), and 1,20-(ethylamino)-5,10,15-triazanonadecane (BE-4444). These compounds have been shown to possess a wide variety of therapeutic effects and illustrate that small structural changes in alkylpolyamine analogs can result in surprisingly significant changes in biological activity.

A major advantage of the *bis*(ethyl)polyamines lies in the fact that their synthesis is extremely straightforward and depends only on the availability of the appropriate parent polyamine backbone. Functionalization of the terminal nitrogens can be readily

accomplished by protecting all of the nitrogens in the parent chain with a tosyl or mesityl protecting group, producing an intermediate that possesses acidic hydrogens at the terminal nitrogen moieties, and unreactive central nitrogens. Alkylation of the terminal nitrogens is then accomplished by a sodium hydride-catalyzed reaction with ethyl bromide followed by deprotection of the nitrogens and recrystallization from ethanol/water *(23–25)*. The *N*,*N*′-*bis*(ethyl)polyamines are readily transported into mammalian cells by the same transport mechanism as the natural polyamines *(26)*. Treatment of mammalian cells with these analogs leads to a reduction of putrescine, spermidine, and spermine, downregulation of ODC and AdoMetDC, and, depending on the cell lines used, cytostasis or cytotoxicity *(23,26)*. These effects are accompanied by a tremendous induction of SSAT activity, in some cases as much as 1000-fold. Preliminary structure/activity correlations, based only on data from the symmetrically alkylated polyamine analogs, suggested that monoalkylation at both terminal nitrogens of spermidine or spermine was important for optimal antiproliferative activity, and that alkylation at an internal nitrogen reduced in vitro activity *(23)*. It was further determined that the greatest induction of SSAT was dependent on the presence of "protected" aminopropyl or aminobutyl moieties *(27–29)*. Adding terminal nitrogen *bis*(alkyl) substituents larger than ethyl resulted in a dramatic reduction in antitumor activity *(23,30,31)*. Compounds with a 3-3-3 carbon skeleton were more effective than the corresponding 3-4-3 analogs, and spermine-like compounds (3-3-3 or 3-4-3) are more effective that spermidine-like analogs (3-3 or 3-4). However, these data were collected using only symmetrically substituted *bis*-alkylpolyamines, and as a result, only *bis*(ethyl)-substituted analogs were advanced to clinical trials. Among these analogs, the most promising were N^1,N^{11}-*bis*(ethyl)norspermine (BENSpm;Fig. 6), which has a 3-3-3 backbone; N^1,N^{14}-*bis*(ethyl)norspermine (BEHSpm;Fig. 6), which has a 4-4-4 carbon skeleton; and 1,20-*bis*(ethylamino)-5,10,15-triazanonadecane (BE-4444;Fig. 6), which has a 4-4-4-4 architecture. Interestingly, BEHSpm proved to be useful as an antidiarrheal agent *(32)* and was advanced to clinical trials for this indication.

BENSpm has shown exceptional promise as an antitumor agent in both in vitro and in vivo studies. Early studies indicated that BENSpm was an effective antitumor agent in cultured human pancreatic adenocarcinoma cells and xenografts, human MALME-3 melanoma xenografts, melanocytes, human bladder cancer cells, and ovarian carcinoma tumor cells *(4)*. In $CaCO_2$ colon cancer cells, the analog causes induction of SSAT, downregulation of ODC, and depletion of cellular polyamines, resulting in cytotoxicity *(33)*. In addition, SSAT induction appears to be the common event leading to cytotoxicity in non-small-cell lung (SCLC) tumor explants *(34)*. Presumably because of the proprietary nature of data concerning BENSpm, no human clinical trials involving the compound have been published, although it is known anecdotally that these trials were initiated. The closely related analog BEHSpm (Fig. 6) does not show similar promise as an antitumor agent *(35)*, but is being developed as an effective treatment for AIDS-related diarrhea *(32,35,36)*. The potent antidiarrheal activity of BEHSpm has been demonstrated in several animal models, and in human clinical trials involving patients with AIDS-related diarrhea. A limited structure/activity study was conducted, and the closely related analog N^1,N^{12}-*bis*(isopropyl)spermine (BIPSpm;Fig. 6) proved

to be the most active antidiarrheal in the series *(32)*. The pharmacokinetics of BENSpm *(37)* and BEHSpm *(38)* for in vivo metabolism have been described. BENSpm is metabolized by *N*-de-ethylation and stepwise removal of aminopropyl equivalents by SSAT and PAO, with a half-life of 73 min. BEHSpm was metabolized almost exclusively to homospermine, which cannot serve as a substrate for SSAT and thus persists in tissues for a period of weeks (liver $T_{1/2} = 15.4$ d). Chronic administration of BEHSpm results in tissue accumulation of the analog and homospermine, resulting in disruption of normal polyamine metabolism. The metabolically programmed alkylpolyamine 3,12-dihydroxyBEHSpm (Fig. 6) retained the antidiarrheal activity of the parent BEHSpm, and exhibited a significantly diminished tissue half-life, presumably from the metabolic "handles" provided by the hydroxyl groups *(39)*. The alkylpolyamine BE-4444 (Fig. 6) was originally designed based on the hypothesis that analogs with chain lengths different from spermine could exhibit enhanced binding to DNA and thus exert antiproliferative effects *(40)*. BE-4444 has been shown to be effective in cultured U-251, MG, SF-126, and SF-188 brain tumor cells at a concentration of 5 μ*M* *(40,41)* and against DU-145, LNCaP, and PC-3 prostate cancer cells in vitro and in vivo *(42)*.

Specific alterations to the polyamine backbone structure of BESpm, BEHSpm, and BE-4444 has resulted in a series of "second-generation" *bis*(ethyl)polyamines with impressive antitumor and antiparasitic activity. Restriction of rotation in the central region of the polyamine chain in BESpm by including a cis- and trans-cyclopropyl or cyclobutyl ring, a cis- and trans-double bond, a triple bond, and a 1,2-disubstituted aromatic ring produced analogs (Fig. 7) with varying antitumor activity in a panel of human tumor cell lines (A549, HT-29, U251MG, DU145, PC-3, and MCF7) *(43)*. There was little difference between the cis and trans isomers in the cyclopropyl, cyclobutyl, and double bond-containing analogs, and the triple bond and aromatic substituents rendered the resulting analogs inactive. All of the analogs were imported by the polyamine transport system, suggesting that the lack of activity was because of diminished DNA binding affinity. In like fashion, insertion of a central dimethylsilane group resulted in a significant decrease in growth inhibition when compared with the *bis*(ethyl)polyamine analogs *(44)*. It was later found that the *bis*(ethyl)spermine analog N^1,N^{12}-*bis*(ethyl)-*cis*-6,7-dehydrospermine (SL-11047;Fig. 8) was an effective treatment for *Cryptosporidium parvum* infections, producing cures in a murine model *(45)*. By contrast, the 4-4-4 (homospermine) analog SL-11093 (Fig. 8), which contains a trans cyclopropyl moiety in the central region, was an effective antitumor agent in vitro, and in vivo against DU-145 nude mouse xenografts *(46)*. Compounds with a 4-4-4 or 4-4-4-4-4 backbone that featured trans-cyclopropyl or a trans-cyclobutyl moieties in noncentral regions of the chain were more active in vitro against prostate tumor cell lines (LnCap, DU145, DUPRO, and PC-3), and inclusion of a cis unsaturation in one of the terminal aminobutyl groups also enhanced activity, presumably by enhancing DNA binding *(47)*. The trans-*bis*-cyclopropyl analogs *bis*(cyclopropane)tetramine A and *bis*(cyclopropyl)hexamine B (Fig. 8) were effective antitumor agents against DuPro and DU-145 prostate tumor cells in vitro *(48)*. In general, structural modifications to homospermine-like backbones that are analogous to those made to the BESpm backbone (i.e., cis- and trans-cyclopropyl, cyclobutyl, and double-bond moieties) afforded

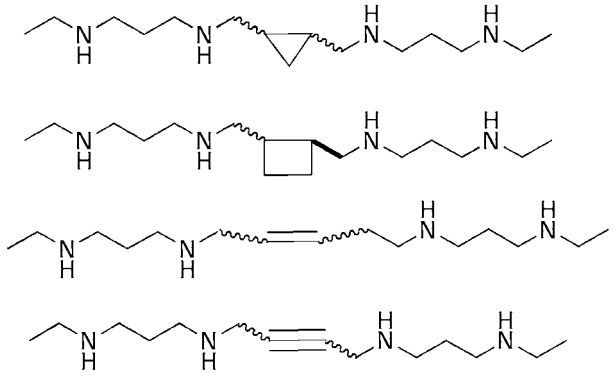

Fig. 7. Conformationally restricted analogs of BESpm.

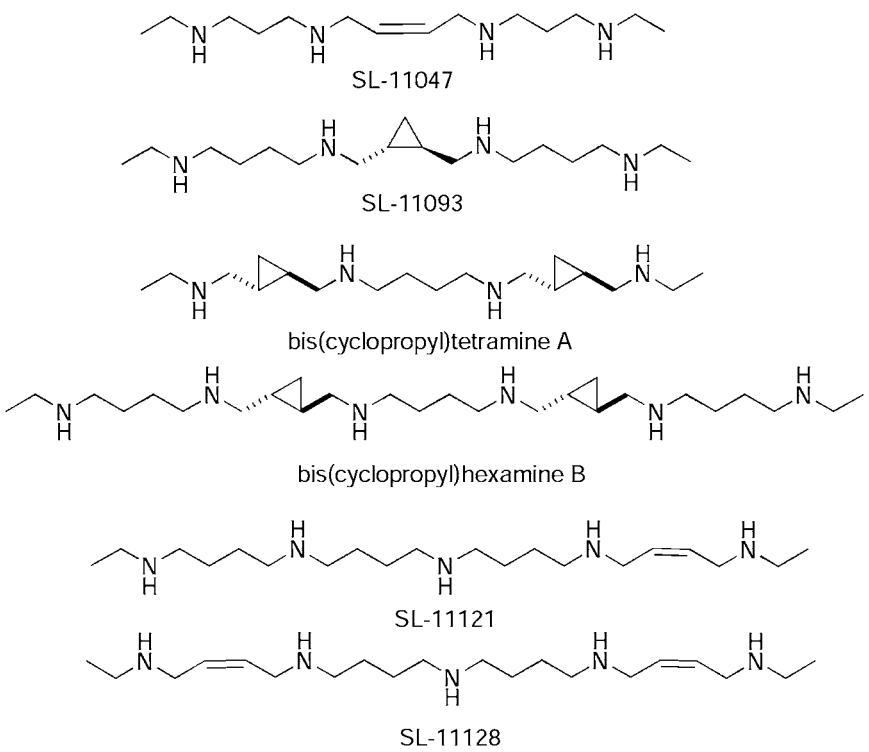

Fig. 8. Conformationally restricted *bis*(ethyl)polyamine analogs with enhanced antitumor activity against prostate tumor cells in culture.

analogs with enhanced antitumor activity and diminished systemic toxicity. In addition, insertion of a cis double bond into the terminal aminobutyl moieties of BE-4444

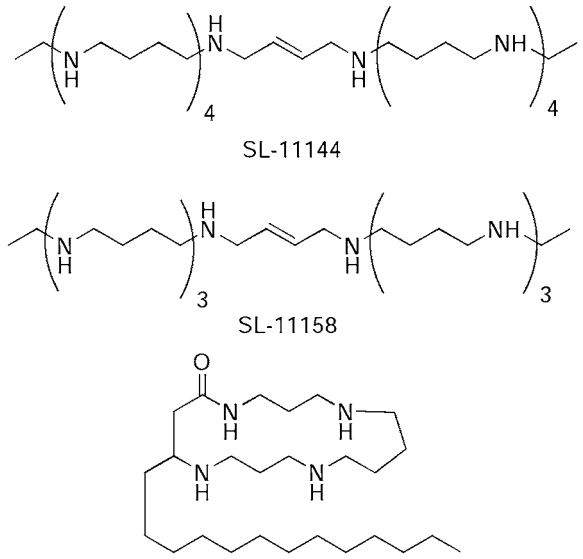

Fig. 9. Structures of oligamines and macrocyclic polyamines with antitumor activity.

(i.e., SL-11121 and SL-11128, Fig. 8) also affords analogs that are equipotent to BE-4444 with respect to ID_{50} values, but that are an order of magnitude more cytotoxic in a dose-response study *(49)*.

Recently, a series of *bis*(ethyl)oligamine analogs have been described that show promise as potential chemotherapeutic agents. In the limited series of oligamines that were evaluated, the decamine SL-11144 and the octamine SL-11158 (Fig. 9) proved to be most growth inhibitory against a panel of prostate tumor cells in vitro (LnCap, DU-145, DuPro, and PC-3). Not surprisingly, their activity roughly correlated with their ability to aggregate DNA *(50)*. It has been shown that macrocyclic polyamines known as budmunchiamines act as potent antitumor agents by virtue of their ability to selectively deplete adenosine triphosphate. Based on this observation, a series of five macrocyclic polyamines with the representative structure shown in Fig. 9 were synthesized and evaluated as antitumor agents in the DuPro and PC-3 prostate cell lines *(51)*. All five of these analogs were readily imported by cells and caused a dramatic depletion of cellular polyamines. These compounds also proved to be cytotoxic in the tumor lines tested, and the degree of cytotoxicity roughly correlated to their ability to deplete adenosine triphosphate.

One unusual characteristic of the *bis*(ethyl)polyamines is their ability to produce cell type-specific cytotoxicity in two representative lung cancer cell types, NCI H157 non-SCLC and H82 SCLC. Soon after the first alkylpolkyamines were described, it was shown that the *bis*(ethyl)polyamines were cytotoxic to DFMO-resistant H157 cells *(29)* that are clinically characterized as being refractory to all treatment modalities. By contrast, the *bis*(ethyl)polyamines are relatively ineffective against DFMO-sensitive SCLC lines. The mechanisms underlying the observed differential sensitivities are still being elucidated, but it was noted that unusually high induction of SSAT (in some cases

>1000 fold) in cell types that respond to *bis*(ethyl)polyamine analogs, but not in the refractory cell lines, and a lack of SSAT induction in the refractory SCLC line H82 *(15,29)*. In H157 cells in culture, the induction of SSAT correlated with a time- and dose-dependent increase in SSAT steady-state messenger RNA levels, suggesting a transcriptional level of control over SSAT synthesis. The correlation between high induction of the SSAT activity and cytotoxicity was subsequently demonstrated for other examples of human malignancies, including human melanomas.

2.2. Unsymmetrically Substituted Alkylpolyamine Analogs

Structure activity studies involving the *bis*(ethyl)polyamines revealed much about the role of charge and flexibility in the polyamine backbone structure. However, the most useful compounds were symmetrically substituted with ethyl groups at the terminal nitrogens, and it was concluded that substituents of greater size than ethyl would render a molecule inactive. It was clear that unsymmetrically substituted alkylpolyamines needed to be synthesized to determine the optimal substituent pattern for the terminal nitrogens, and to explore the chemical space surrounding the terminal alkyl groups. Compounds that possessed unsymmetrically substituted terminal nitrogens were first described in 1993 *(52)*, the first of which being N^1-propargyl-N^{11}-ethylnorspermine (PENSpm) and N^1-cyclopropylmethyl-N^{11}-ethylnorspermine (CPENSpm), which are shown in Fig. 10. In general, the synthesis of these and other unsymmetrically substituted analogs is more difficult because it requires selective protection and deprotection of the internal and external nitrogens. Preliminary results indicated that both PENSpm (IC_{50} = 1.1 μ*M*) and CPENSpm (IC_{50} = 1.1 μ*M*) were as active or more active than BESpm, both with respect to SSAT induction and cytotoxicity in H157 cells in culture. These analogs also retained the cell type-specific cytotoxic activity observed after treatment with BESpm, and their activity was directly correlated to their ability to induce SSAT. This increase in SSAT activity was accompanied by a cell-specific increase in steady-state SSAT messenger RNA that was similar in magnitude to that observed after treatment of the H157 cells with BESpm or BENSpm. These data suggest a similar mechanism of induction of SSAT for these compounds, and supported the hypothesis that there may be a functional relationship between cytotoxicity and SSAT induction in the non-SCLC and SCLC cell lines. A third compound in this series, N^1-cycloheptylmethyl-N^{11}-ethylnorspermine (CHENSpm, Fig. 10), was subsequently synthesized and evaluated *(4)* and was found to retain antitumor activity (IC_{50} = 0.25 μ*M* against non-SCLC cells) while producing a less-pronounced cell type–specificity. However, when the induction of SSAT and polyamine levels in the H157 cell line were measured, the analog showed striking differences from the parent analog BESpm. Treatment of H157 cells with 10 μ*M* BESpm depleted the natural polyamines to undetectable levels and produced a 2849-fold increase in SSAT activity. Under these conditions, the only polyamine that was present in the cell in significant amounts was the analog itself, a response that is typical among SSAT-inducing alkylpolyamines. Surprisingly, treatment with 10 μ*M* CHENSpm had almost no effect on the levels of putrescine, spermidine, and spermine, and caused only a 15-fold induction of SSAT activity *(4)*. These data suggested that the cytotoxic effects produced by BENSpm,

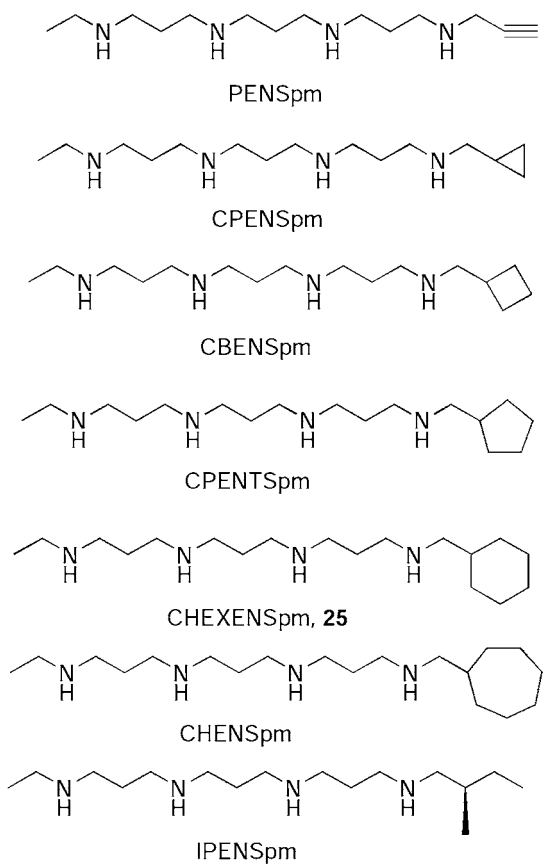

Fig. 10. The unsymmetrically substituted alkylpolyamines PENSpm, CPENSpm, CBENSpm, CPENTSpm, CHEXENSpm, CHENSpm, and IPENSpm.

PENSpm, and CPENSpm in H157 cells could be mediated by different cellular mechanisms than the effects produced by CHENSpm. In response to these findings, three additional compounds were synthesized that possessed substituents containing the intervening ring sizes (CBENSpm, CPENTSpm, and CHEXENSpm), as shown in Fig. 10. All three analogs were generally cytotoxic in both the H157 and H82 cell lines, but there was no correlation between the induction of SSAT and the IC_{50} value in the H157 line, as shown in Fig. 11. In this cycloalkyl series, the induction of SSAT decreased dramatically as a function of ring size, whereas the IC_{50} values were remarkably constant (0.4–0.7 μ*M*). These data clearly support the contention that there are at least two mechanisms by which unsymmetrically substituted alkylpolyamines produce cytotoxicity in H157 non-SCLC cells.

Based on data obtained for unsymmetrically substituted alkylpolyamine analogs, it is evident that the structure/activity relationships (SAR) of these polyamine analogs is

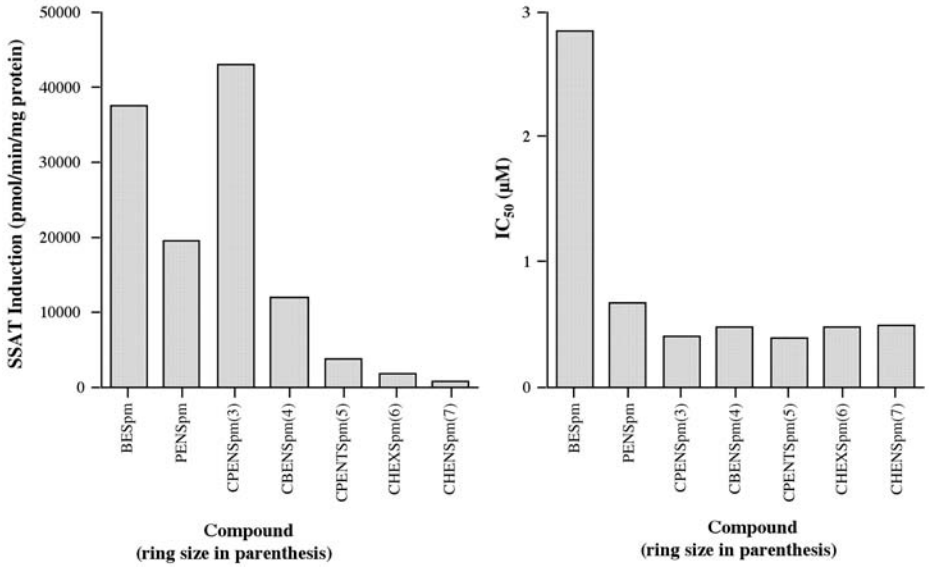

Fig. 11. 96 h SSAT induction and IC_{50} values in the NCI H157 cell line for selected alkylpolyamine analogs.

more complex than originally postulated. Optimal activity is obtained from molecules that possess secondary terminal nitrogens, but only one of the two terminal nitrogens of the backbone need be substituted with a small alkyl group. Structurally similar compounds, like CPENSpm and CHENSpm, which differ only in the number of carbons in the cycloalkyl substituents, appear to inhibit cell growth by completely different mechanisms. It has also been noted that compounds that significantly induce SSAT tend to be more cell type-specific in their activity than those that do not *(4)*.

In addition to the lung cancer model, unsymmetrically substituted polyamine analogs have been evaluated in prostate cancer model systems *(53)*. CPENSpm and CHENSpm were cytotoxic to the DU145 cell line at concentrations ≥1 μ*M* with significant accumulation of each analog, and CHENSpm was found to be cytotoxic to the DU145, PC-3, and LnCap cell lines at 30 μ*M*. Although the effects of these analogs on the polyamine metabolic pathway appear to be modest in the prostate lines, the cytotoxicity produced in these cells at low concentrations encourages further study.

The data outlined here suggest that unsymmetrically substituted alkylpolyamines can exhibit varying degrees of SSAT induction based on the size of their terminal alkyl substituents. The SAR model for unsymmetrically substituted alkylpolyamines that superinduce SSAT is shown in Fig. 12A *(4)*. Analogs in this subclass generally possess a 3-3-3 or 3-4-3 carbon skeleton and a *bis*(alkyl) substitution pattern on the terminal nitrogens. Unsymmetrically substituted analogs induce as well or better than the parent analogs BESpm and BENSpm. When R_1 is ethyl, R_2 can vary in size from small (e.g., ethyl, cyclopropylmethyl) to medium size; however, the size of only one of the substituents can be increased beyond ethyl. The central nitrogens are separated by 5.0–5.8 Å,

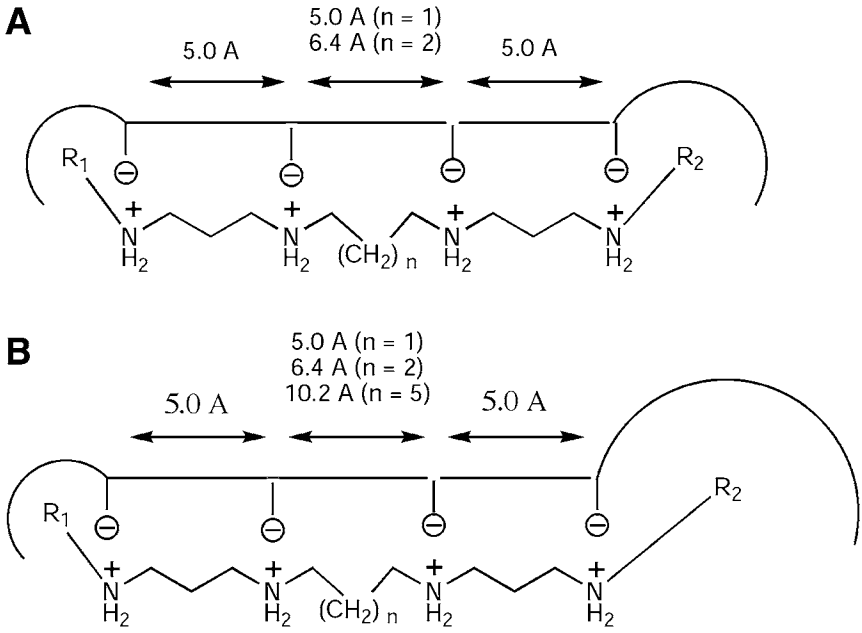

Fig. 12. SAR models for alkylpolyamine analogs. **(A)** SSAT induction-dependent antitumor agents. **(B)** SSAT induction-dependent alkylpolyamine antitumor agents and antiparasitic agents.

and each terminal nitrogen is 5.0 Å from the adjacent central nitrogen. The binding pocket for R_1 will accept only a small alkyl group, whereas the R_2 binding pocket can accommodate medium sized groups up to the size of cyclopentylmethyl. Both spermine and spermidine analogs can bind to this site, because both types of analogs are capable of superinducing SSAT. The cytotoxicity of agents that fit these criteria can be directly related to the superinduction of SSAT.

The model for the design of SSAT induction-independent alkylpolyamine analogs is shown in Fig. 12B *(4)*. The requirement for *bis*(alkyl) substitution remains, but the binding pockets for R_1 and R_2 seem to be less restrictive. The most active analogs have a small (ethyl) or medium sized R_1 and a large R_2 (e.g., cycloheptylmethyl or cyclohexylmethyl). It is required that one of the alkyl groups must be small (e.g., ethyl) whereas the other must be larger than cyclopentylmethyl. Agents with two large terminal alkyl substituents may possess activity, but are not likely to superinduce SSAT. Active analogs with a 3-3-3 and with a 3-7-3 carbon skeleton have been identified, indicating that active compounds may be synthesized with *n* varying between 1 and 5. Thus the requirement for the intermediate chain seems to be less restrictive than in the SSAT inducer series. However, steric bulk on the intermediate chain is not well tolerated. These data suggest an effector site which has internal anionic sites between 5 and 10 Å apart, and terminal anionic sites that are roughly 5 Å away from the respective internal anionic sites. The data suggest that agents that fit these criteria produce cytotoxicity through an as yet undetermined pathway. It has also been noted that analogs

with a 3-7-3 architecture can act as antiparasitic agents, a topic that is beyond the scope of this chapter.

3. Mechanisms for Alkylpolyamine-Induced Cytoxicity

It is clear that alkylpolyamines are capable of producing rapid cytotoxicity in lung and prostate tumor cell lines, but the mechanistic aspects of these effects must still be fully elucidated. However, the induction of programmed cell death (PCD) appears to be a common result after treatment with alkylpolyamines from both structural classes mentioned previously. This effect was first observed in the MCF-7 and MDA-MB-468 breast cancer lines, and later in the H-157 non-SCLC human lung tumor cell line after treatment with CPENSpm *(54)*. In the case of the breast cancer lines, greater than 90% growth inhibition was observed after prolonged treatment with CPENSpm in each of six cell lines tested. The IC_{50} values for inhibition by CPENSpm in these six breast tumor lines ranged from 0.2 to 1.3 μ*M*. In the breast cancer lines MCF-7 and MDA-468, high molecular weight DNA fragmentation and formation of oligonucleosomal-sized fragments were observed as early as 72 h at a 10 μ*M* concentration and after 96 h with as little as 1 μ*M*. Similar results were observed in other breast cancer lines including: T47D, Zr-75-1, MDA 231, and Hs578t. In the case of the NCI H157 lung cancer model, PCD was found to occur at earlier exposure times than observed in the breast tumor lines *(53)*. High molecular weight (≥50 kbp) DNA fragmentation was observed after 24 h exposure to 10 μ*M* CPENSpm. Similar results were observed with 10 μ*M* BENSpm, but only after 48 h, although the initiation of PCD in Chinese hamster ovary (CHO) cells is quite rapid at high concentrations of the analog *(4)*. Although these results clearly indicate that the unsymmetrically substituted analogs induce PCD, the underlying cellular mechanism(s) had not been elucidated.

Acetylation and subsequent oxidation of polyamines by the SSAT/ PAO pathway is known to produce H_2O_2 as a byproduct. During superinduction of SSAT, PCD produced by CPENSpm in H157 cells may result from oxidative stress resulting from H_2O_2 overproduction. When catalase is added in combination with CPENSpm, high molecular weight DNA fragmentation and early fragmentation of the nuclei are greatly reduced *(55)*. Inhibition of PAO by the specific inhibitor *N,N′-bis*(2,3-butadienyl)-1,4-butane-diamine (MDL 72527) resulted in a significant reduction in the formation of high molecular weight DNA, and similarly reduced the number of apoptotic nuclei formed after CPENSpm treatment. These results strongly suggest that H_2O_2 production by PAO has a role in compound CPENSpm-induced cytotoxicity in H157 cells. Catalase or MDL 72527 had no effect on the formation of high molecular weight DNA fragments or apoptotic bodies when coadministered with CHENSpm, supporting the contention that CPENSpm and CHENSpm produce apoptosis by different mechanisms. Treatment of wild-type H157 cells with both CPENSpm and CHENSpm leads to the activation of caspase-3 and cleavage of poly (adenosine 5′-diphosphate-ribose) polymerase *(4)*. In H157 cells that overexpress Bcl-2, many of the known steps of the cell death program, including caspase-3 activation, poly (adenosine 5′-diphosphate-ribose) polymerase cleavage, and the release of cytochrome c from the mitochondria, were blocked in analog-treated H157 cells. However, the overexpression of Bcl-2 was

only able to alter the kinetics of PCD, not completely block it. Thus, both CPENSpm and CHENSpm are capable of inducing PCD in a caspase-3-independent manner.

Several groups have demonstrated that some polyamine analogs (e.g., BE-4444, CHENSpm) that do not superinduce SSAT can still produce PCD. Consistent with this hypothesis is the observation that CPENSpm and CHENSpm have dramatically different effects on the cell cycle *(56)*. After 24 h treatment of H157 non-SCLC with 10 μ*M* CPENSpm, no significant effects on cell cycle are observed by flow cytometric analysis. However, under the same conditions, 10 μ*M* CHENSpm produces a dramatic G_2/M cell cycle block in normal and Bcl-2-overexpressing H157 cells *(4)*. The analog *S*-1-{*N*-[(2-methyl)-1-butyl]amino}-11-[*N*-(ethyl)amino]-4,8-diazaundecane (IPENSpm;Fig. 10) was subsequently found to produce a similar G_2/M cell-cycle arrest *(57)*. All three analogs demonstrated similar cytotoxic effects in the human non-SCLC line, NCI H157, where they were found to be cytotoxic at concentrations greater than 0.1 μ*M*, but significant induction of SSAT activity was only observed in cells treated with CPENSpm. The effects of all three compounds on the cell cycle progress were analyzed by flow cytometry after a 24-h exposure to 10 μ*M* of each compound. As previously observed, CPENSpm treatment had no significant effect on the cell cycle. However, both CPENSpm and IPENSpm produced a significant G_2/M cell-cycle arrest and a concurrent decrease in the G_1 fraction. All three analogs, as well as the natural tetraamine, spermine, stimulate tubulin polymerization in the absence of microtubule-associated proteins and other polymerization stimulants, and the rate of polymerization was greatest in the case of CHENSpm (4.9 times faster than spermine). In the presence of microtubule-associated protein-rich tubulin, CHENSpm remained the most effective promoter of tubulin polymerization, whereas CPENSpm and spermine showed significant decreases in their ability to effect tubulin polymerization. These data suggest that CPENSpm, but not CHENSpm, are possibly competing for binding at the site normally occupied by microtubule-associated proteins.

The symmetrically and unsymmetrically substituted alkylpolyamines described have been of great value in determining the mechanisms of analog-induced cytotoxicity. However, the alkyl substituents in these molecules are representative of only a minute portion of the available chemical diversity for the terminal alkyl substituents. Recently, more than 200 alkylpolyamines have been synthesized and evaluated as antitumor agents in an effort to refine the SAR model described in Fig. 12. Preliminary biological evaluation of these analogs was conducted using a high-throughput screen based on 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT cell) viability determination in NCI H157 lung tumor cells. The structures of several of these analogs that demonstrate new structural directions to exploit are shown in Fig. 13, along with their IC_{50} values in the MTT high-throughput screen. The compounds designated 39-TDW-47C, 39-TDW-12C, and 46-TDW-34C were selected for in vivo studies in an A549 lung tumor xenograft model. Preliminary studies indicate that all three of these analogs are effective in limiting tumor growth in the xenograft model. It is important to note that the compounds shown in Fig. 13 contain structural features that have not previously been included in polyamine analogs described. The data indicate that it is possible to synthesize active alkylpolyamines that contain aralkyl substituents, heteroatoms,

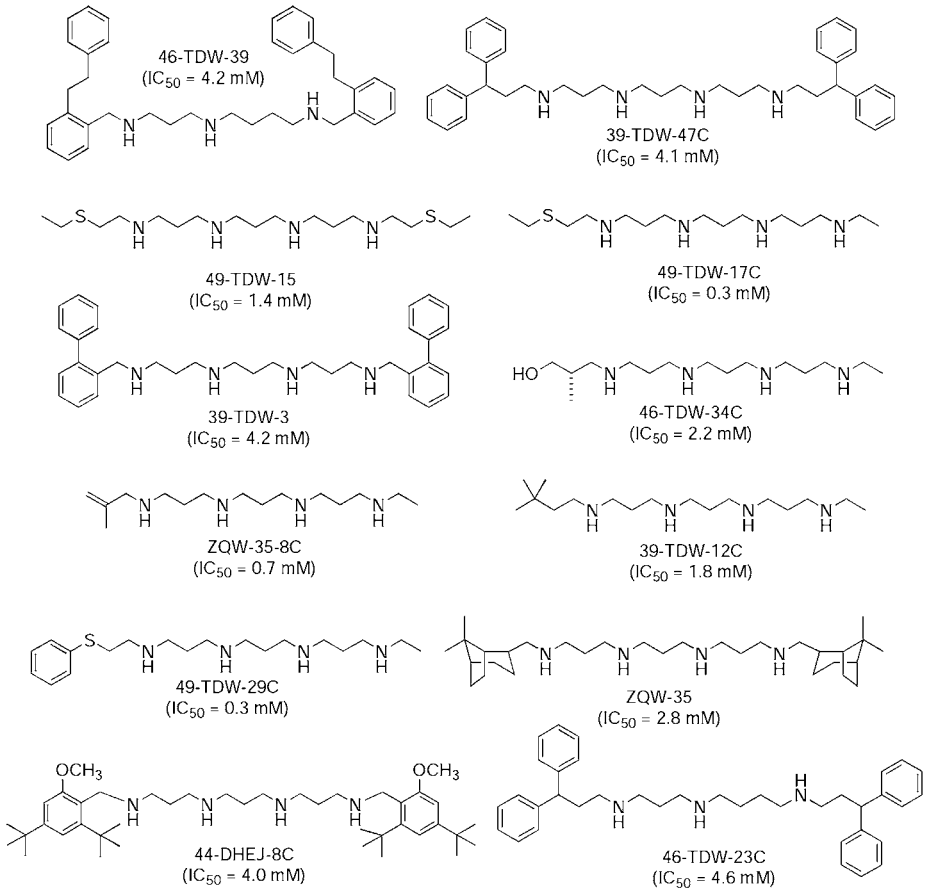

Fig. 13. Novel, structurally diverse alkylpolyamine analogs.

and unsaturations in the terminal alkyl substituents. Additional analogs in this series are being synthesized and used to determine the structural requirements for binding at the various alkylpolyamine effector sites.

4. Future Directions for Polyamine Drug Discovery

As recently as 20 yr ago, the polyamine biosynthetic pathway was still being elucidated and the enzymes were being characterized. Drug discovery efforts were focused on finding specific inhibitors for these enzymes and at determining the cellular consequences of selective depletion of individual polyamines. The polyamine metabolic pathway is now well defined, the enzymes have been characterized, cloned, and expressed in bacterial vectors, and, in the case of AdoMet-DC, the crystal structure of the enzyme is known *(58)*. These research advances have resulted in one marketed agent (i.e., DFMO), two agents that were not developed because of the economic status of the target population

(AbeAdo, MDL 27695), and two or three agents that have the potential will be marketed, and have been or will be studied in human clinical trials as antitumor or antidiarrheal agents (BENSpm, *bis*(ethyl)-4444 and BEHSpm). More recent data suggest that some of the antitumor effects attributed to alkylpolyamines are mediated at sites that are independent of the metabolic enzymes. In terms of drug discovery and development, there are multiple avenues through which polyamine analogs may prove to be useful therapeutic agents. Inhibition of the transport system, which mediates both influx and efflux of polyamines from the cell, may prove to be a more reliable way to disrupt polyamine metabolism than selective inhibition of the individual metabolic enzymes. There are a variety of new polyamine–protein binding interactions (e.g., nuclear factor kB, p53 gene expression, the c-myc pathway, expression of caspase-3) that could be validated as targets for novel polyamine-based antitumor agents. Such interactions could be viewed as targets for polyamine analogs that are either chemotherapeutic or chemopreventative. As these targets are refined and validated, second-generation agents that target the functions of the natural polyamines can be rationally designed to bind to these nonenzymatic polyamine binding sites. During the next few years, many research groups will be working toward defining these targets and developing agents that specifically bind to these sites and modulate their function. Although this chapter focuses on the use of polyamine analogs as antitumor agents, there are a number of groups developing analogs that target other known polyamine functions (e.g., analog binding to the *N*-methyl-D-aspartate (NMDA) receptor, polyamine analogs as antiparasitic agents, polyamines as vectors for gene and drug delivery). The ubiquitous nature of the polyamines, and the wide variety of effects they produce, virtually guarantees that new polyamine effector sites will be discovered, and that these sites will provide new avenues for drug design and development.

References

1. Bergeron, R. J., McManis, J. S., Weimar, W. R., et al. (1995) The role of charge in polyamine analog recognition. *J. Med. Chem.* **38,** 2278–2285.
2. Cohen, S. A. (ed.) (1998) *Guide to the Polyamines.* Oxford University Press, New York.
3. Sunkara, P. S., Baylin, S. B., and Luk, G. D. (1987) Inhibitors of polyamine biosynthesis: Cellular *in vivo* effects on tumor proliferation. In: *Inhibition of Polyamine Metabolism: Biological Significance and Basis for New Therapies* (McCann, P. P., Pegg, A. E., and Sjoerdsma, A., eds.), Academic Press, New York, pp. 121–140.
4. Casero, Jr., R. A. and Woster, P. M. (2001) Terminally alkylated polyamine analogues as chemotherapeutic agents. *J. Med. Chem.* **44,** 1–29.
5. Gerner, E. W. and Meyskens, F. L. (2004) Polyamines and cancer: Old molecules, new understanding. *Nat. Rev. Cancer* **4,** 781–792.
6. Pegg, A. E. and McCann, P. P. (1992) S-adenosylmethionine decarboxylase as an enzyme target for therapy. *Pharmacol. Ther.* **56,** 359–377.
7. Pegg, A. E. (1988) Polyamine metabolism and its importance in neoplastic growth and as a target for chemotherapy. *Cancer Res.* **48,** 759–774.
8. Murikami, Y., Matsufuji, S., Kameji, T., et al. (1992) Ornithine decarboxylase is degraded by the 26S proteasome without ubiquitination. *Nature* **360,** 597–599.
9. Metcalf, B. W., Bey, P., Danzin, C., Jung, M. J., Casara, P., and Vevert, J. P. (1978) Catalytic, irreversible inhibition of mammalian ornithine decarboxylase (E.C. 4.1.1.17) by substrate and product analogues. *J. Am. Chem. Soc.* **100,** 2551–2553.

10. Tang, K.-C., Mariuzza, R., and Coward, J.K. (1981) Synthesis and evaluation of some stable multisubstrate adducts as specific inhibitors of spermidine synthase. *J. Med. Chem.* **24,** 1277–1284.
11. Woster, P. M., Black, A. Y., Duff, K. J., Coward, J. K., and Pegg, A. E. (1989) Synthesis and biological evaluation of S-adenosyl-1,12-diamino-3-thio-9-azadodecane, a multisubstrate adduct inhibitor of spermine synthase. *J. Med. Chem.* **32,** 1300–1307.
12. Recsei, P. A. and Snell, E. E. (1984) Pyruvoyl enzymes. *Ann. Rev. Biochem.* **53,** 357–387.
13. Danzin, C., Marchal, P., and Casara, P. (1990) Irreversible inhibition of rat S-adenosylmethionine decarboxylase by 5′{[(Z)-4-amino-2-butenyl]methylamino}-5′-deoxyadenosine. *Biochem. Pharmacol.* **40,** 1499–1503.
14. Marchant, P., Dredar, S., Manneh, V., et al. (1989) A selective inhibitor of N^8-acetylspermidine deacetylation in mice and HeLa cells without effects on histone deacetylation. *Arch. Biochem. Biophys.* **15,** 128–136.
15. Casero, R. A. and Pegg, A. E. (1993) Spermidine/spermine-N^1-acetyltransferase—the turning point in polyamine metabolism. *FASEB J.* **7,** 653–661.
16. Cullis, P. M., Green, R. E., Merson-Davies, L., and Travis, N. G. (1998) Chemical highlights of polyamine transport. *Biochem. Soc. Trans.* **26,** 595–601.
17. Seiler, N., Delcros, J. G., and Moulinoux, J. P. (1996) Polyamine transport in mammalian cells. An update. *Int. J. Biochem. Cell Biol.* **8,** 843–861.
18. Kashiwagi, K., Endo, H., Kobayashi, H., Takio, K., and Igarashi, K. (1995) Spermidine-preferential uptake system in *Escherichia coli*. ATP hydrolysis by PotA protein and its association with membrane. *J. Biol. Chem.* **270,** 25377–25382.
19. Seiler, N. (2003) Thirty years of polyamine-related approaches to cancer therapy. Retrospect and prospect. Part 2. Structural analogues and derivatives. *Curr. Drug Targets* **7,** 565–585.
20. Edwards, M. L., Prakash, N. J., Stemerick, D. M., et al. (1990) Polyamine analogues with antitumor activity. *J. Med. Chem.* **33,** 1369–1375.
21. Edwards, M. L., Snyder, R. D., and Stemerick, D. M. (1991) Synthesis and DNA-binding properties of polyamine analogues. *J. Med. Chem.* **34,** 2414–2420.
22. Porter, C. W. and Sufrin, J. (1986) Interference with polyamine biosynthesis and/or function by analogues of polyamines or methionine as a potential anticancer chemotherapeutic strategy. *Anticancer Res.* **6,** 525–542.
23. Bergeron, R. J., Neims, A. H., McManis, J. S., et al. (1988) Synthetic polyamine analogues as antineoplastics. *J. Med. Chem.* **31,** 1183–1190.
24. Yajima, H., Takeyama, M., Kanaki, J., Nishimura, O., and Fujino, M. (1978) Studies on peptides. LXXX. N^G-Mesitylene-2-sulfonylarginine. *Chem. Pharm. Bull.* **26,** 3752–3757.
25. Roemmele, R. C., and Rappoport, H. (1998) Removal of N-arylsulfonyl groups from hydroxy α-amino acids. *J. Org. Chem.* **53,** 2367–2371.
26. Porter, C. W., Berger, F. G., Pegg, A. E., Ganis, B., and Bergeron, R. J. (1987) Regulation of ornithine decarboxylase activity by spermidine or the spermidine analog N^1,N^8-bis(ethyl)spermidine (BES). *Biochem. J.* **242,** 433–440.
27. Casero, R. A., Ervin, S. J., Celano, P., Baylin, S. B., and Bergeron, R. J. (1989) Differential response to treatment with *bis*(ethyl)polyamine analogues between human small cell lung carcinoma and undifferentiated large cell lung carcinoma in culture. *Cancer Res.* **49,** 639–643.
28. Porter, C. W., Ganis, B., Libby, P. R., and Bergeron, R. J. (1991) Correlations between polyamine analogue-induced increases in spermidine/spermine-N1-acetyltransferase activity, polyamine pool depletion, and growth inhibition in human melanoma cell lines. *Cancer Res.* **51,** 3715–3720.

29. Casero, R. A., Mank, A. R., Xiao, L., Smith, J., Bergeron, R. J., and Celano, P. (1992) Steady-state messenger RNA and activity correlates with sensitivity to N^1,N^{12}-*bis*(ethyl)spermine in human cell lines representing the major forms of lung cancer. *Cancer Res.* **52,** 5359–5363.
30. Bergeron, R. J., Hawthorne, T. R., Vinson, J. R. T., Beck, D. E., Jr., and Ingeno, M. J. (1989) Role of the methylene backbone in the antiproliferative activity of polyamine analogues in L1210 cells. *Cancer Res.* **49,** 2959–2964.
31. Bergeron, R. J., McManis, J. S., Liu, C. Z, et al. (1994) Antiproliferative properties of polyamine analogues: a structure-activity study. *J. Med. Chem.* **37,** 3464–3476.
32. Bergeron, R. J., Wiegand, J., McManis, J. S., et al. (2001) Polyamine analogue antidiarrheals: a structure-activity study. *J. Med. Chem.* **44,** 232–244.
33. Berchtold, C. M., Tamez, P., Kensler, T. W., and Casero, R. A. (1998) Inhibition of cell growth in CaCO2 cells by the polyamine analogue N^1,N^{12}-bis(ethyl)spermine is preceded by a reduction in MYC oncoprotein levels. *J. Cell Physiol.* **174,** 380–386.
34. Gabrielson, E. W., Pegg, A. E., and Casero, R. A. (1999) The induction of spermidine/spermine-N^1-acetyltransferase (SSAT) is a common event in the response of human primary non-small cell lung carcinomas to exposure to a new antitumor polyamine analogue, N^1,N^{11}-bis(ethyl)norspermine. *Clin. Cancer Res.* **5,** 1638–1641.
35. Wilding, G., King, D., Tutsch, K., et al. (2004) Phase I trial of the polyamine analog N^1,N^{14}-diethylhomospermine (DEHSPM) in patients with advanced solid tumors. *Invest. New Drugs.* **22,** 131–8.
36. Sninsky, C. A. and Bergeron, R. (1993) Potent anti-diarrheal activity of a new class of compounds: synthetic analogs of the polyamine pathway. *Gastroenterology* **104,** A584.
37. Bergeron, R. J., Weimar, W. R., Luchetta, G., et al. (1995) Metabolism and pharmacokinetics of N^1,N^{11}-diethylnorspermine. *Drug Metab. Disp.* **23,** 1117–1125.
38. Bergeron, R. J., Weimer, W. R., Luchetta, G., Sninsky, C. A., and Wiegand, J. (1996) Metabolism and pharmacokinetics of N^1,N^{14}-diethylhomospermine. *Drug Metab. Disp.* **24,** 334–343.
39. Bergeron, R. J., Yao, G. W., Yao, H., et al. (1996) Metabolically programmed polyamine analogue antidiarrheals. *J. Med. Chem.* **39,** 2461–2471.
40. Basu, H. S., Pellarin, M., Feuerstein, B. G., Deen, D. F., and Marton, L. J. (1991) Effect of N^1,N^{14}-bis(ethyl)homospermine (BE-4X4) on the growth of U251, MG and SF-188 brain tumor cells. *Int. J. Cancer* **30,** 873–878.
41. Basu, H. S., Pellarin, M., Feuerstein, B. G., Deen, D. F., and Marton, L. J. (1993) Effects of the polyamine analogs BE-3-7-3, 3-8-3, and BE-3-8-3 on human brain tumor cell growth and survival. *Anticancer Res.* **13,** 1525–1532.
42. Jeffers, L., Church, D., Basu, H., Marton, L., and Wilding, G. (1997) Effects of the polyamine analogues BE-4-4-4-4, BE-3-7-3 and BE-3-3-3 on the proliferation of three prostate cancer cell lines. *Cancer Chemother. Pharmacol.* **40,** 172–179.
43. Reddy, V. K., Valasinas, A., Sarkar, A., Basu, H. S., Marton, L. J., and Frydman, B. (1998) Conformationally restricted analogues of ^{1}N,^{12}N-bisethylspermine: synthesis and growth inhibitory effects on human tumor cell lines. *J. Med. Chem.* **41,** 4723–4732.
44. Seiler, N., Delcros, J. G., Vaultier, M., et al. (1996) Bis(7-amino-4-azaheptyl)dimethylsilane and bis(7-ethylamino-4-azaheptyl)dimethylsilane: inhibition of tumor cell growth in vitro and in vivo. *Cancer Res.* **56,** 5624–5630.
45. Waters, W. R., Frydman, B., Marton, L. J., et al. (2000) [^{1}N,^{12}N]Bis(ethyl)-cis-6,7-dehydrospermine: a new drug for treatment and prevention of *Cryptosporidium parvum* infection of mice deficient in T-cell receptor alpha. *Antimicrobial Agents Chemother.* **44,** 2891–2894.

46. Frydman, B., Porter, C. W., Maxuitenko, Y., et al. (2003) A novel polyamine analog (SL-11093) inhibits growth of human prostate tumor xenografts in nude mice. *Cancer Chemother. Pharmacol.* **51,** 488–492.
47. Valasinas, A., Sarkar, A., Reddy, V., Marton, L. J., Basu, H. S., and Frydman, B. (2001) Conformationally restricted analogues of ^{1}N,^{14}N-bisethylhomospermine (BE-4-4-4): Synthesis and growth inhibitory effects on human prostate cancer cells. *J. Med. Chem.* **44,** 390–403.
48. Frydman, B., Blohkin, A. V., Brummel, S., et al. (2003) Cyclopropane-containing polyamine analogues are efficient growth inhibitors of a human prostate tumor xenograft in nude mice. *J. Med. Chem.* **46,** 4586–4600.
49. Reddy, V. K., Sarkar, A., Valasinas, A., Marton, L. J., Basu, H. S., and Frydman, B. (2001) Cis-unsaturated analogues of 3,8,13,18,23-pentaazapentacosane (BE-4-4-4-4): synthesis and growth inhibitory effects on human prostate cancer cell lines. *J. Med. Chem.* **44,** 404–417.
50. Valasinas, A., Reddy, V. K., Blohkin, A. V., et al. (2003) Long-chain polyamines (oligoamines) exhibit strong cytotoxicities against human prostate cancer cells. *Bioorg. Med. Chem.* **11,** 4121–4131.
51. Frydman, B., Bhattacharya, S., Sarkar, A., et al. (2004) Macrocyclic polyamines deplete cellular ATP levels and inhibit cell growth in human prostate cancer cells. *J. Med. Chem.* **47,** 1051–1059.
52. Saab, N. H., West, E. E., Bieszk, N. C., et al. (1993) Synthesis and evaluation of unsymmetrically substituted polyamine analogues as inhibitors of spermidine/spermine-N^{1}-acetyltransferase (SSAT) and as potential antitumor agents. *J. Med. Chem.* **36,** 2998–3004.
53. McCloskey, D. E., Yang, J., Woster, P. M., Davidson, N. E., Casero, R. A., Jr. (1996) Polyamine analogue induction of programmed cell death in human lung tumor cells. *Clin. Cancer Res.* **2,** 441–446.
54. Davidson, N. E., Hahm, H. A., McCloskey, D. E., Woster, P. M., and Casero, R. A. (1999) Clinical aspects of cell death in breast cancer: the polyamine pathway as a new target for treatment. *Endocr. Related Cancer* **6,** 69–73.
55. Ha, H. C., Woster, P. M., Yager, J. D., and Casero, R. A., Jr. (1997) The role of polyamine catabolism in polyamine analogue-induced programmed cell death. *Proc. Natl. Acad. Sci. USA* **94,** 11,557–11,562.
56. Ha, H. C., Woster, P. M., and Casero, R. A., Jr. (1998) Unsymmetrically substituted polyamine analogue induces caspase-independent programmed cell death in Bcl-2 overexpressing cells. *Cancer Res.* **58,** 2711–2714.
57. Webb, H. K., Wu, Z. Q., Sirisoma, N., Ha, H. C., Casero, R. A., Jr., and Woster, P. M. (1999) 1-[N-(Alkyl)amino]-11-[N-(ethyl)amino]-4,8-diazaundecanes: simple synthetic polyamine analogues which differentially alter tubulin polymerization. *J. Med. Chem.* **42,** 1415–1421.
58. Ekstrom, J. L., Mathews, I. I., Stanley, B. A., Pegg, A. E., and Ealick, S. E. (1999) The crystal structure of human S-adenosylmethionine decarboxylase at 2.25 angstroms resolution reveals a novel fold. *Structure (London)* **7,** 583.

2

Acute Increases in Intracellular Putrescine Lead to the Increase in Steady-State Levels of *c-fos, c-jun, RING3,* and *Id-1* mRNAs

Allan A. Ancheta, Leo Hawel, III, and Craig V. Byus

1. Introduction

For a number of years, researchers have noted that cancer cells have higher levels of ornithine decarboxylase (ODC) activity than do the corresponding noncancerous tissues *(1–5)*. Because increases in ODC activity (and subsequently, the polyamines) occur regularly in the cell cycle and are necessary before successful cell division can occur, the exact role of these increased polyamine levels in tumorigenesis was not completely understood. It was unclear whether the increases in ODC and polyamine levels directly participated in the generation on the cancer phenotype, or whether these increases were only a result of the increased cell cycling that is a hallmark of many cancerous cells and tumors *(1–4)*.

This "chicken or egg" conundrum was clearly resolved by the production of a transgenic mouse that overexpressed ODC only in keratinocytes *(5,6)*. By itself, the overexpression of ODC in these animals was unable to cause the appearance of tumors. When the skin of these transgenic mice was treated with a low dose of the mutagen 7,12-dimethylbenz[a]anthracene, an initiator of v-Ha-*ras*, papillomas were readily formed *(7)*. This observation indicated that elevated ODC and the resulting increases in intracellular polyamines could act as a tumor promoter (taking the place of phorbol esters) in this two-stage model of tumorigenesis *(8–10)*. There have been many other studies (many reviewed in this monograph) that have further implicated ODC overexpression and increased polyamines as a causative factor in other experimental models of cancer development and in the etiology of human cancer.

Although the precise mechanisms by which increases in ODC activity (and intracellular polyamine levels) lead to tumor formation are unknown, it appears likely that there must first be an independent initiating event (mutation) that will allow the elevated polyamines to promote tumor growth. This was also demonstrated when a transgenic mouse that expressed the mutant form of v-Ha-*ras* (that normally results from 7,12-dimethylbenz[a]anthracene exposure) was crossed with the ODC overexpressing

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mouse line. Although neither of these two transgenic mouse lines individually bore any tumors, when bred to each other the resulting offspring spontaneously produced papillomas without the need for any further input *(11)*. Additionally, once formed, these tumors could be regressed by the administration of the ODC inhibitor α-difluoromethylornithine (DFMO) in the drinking water of the tumor-bearing mice. When DFMO was removed from the water, the tumors reappeared at the same sites that they had occupied before the DFMO-mediated regression *(12)*. These observations underscore the reversible role that ODC activity and increased polyamine levels play in the promotion and maintenance of these papillomas.

Our hypothesis was that ODC overexpression and sustained elevated polyamine accumulation led to an alteration in net gene expression of a specific subset of genes involved in tumor promotion. Although a number of genes have been shown to be differentially expressed in various tumor tissues, only spermidine/spermine-N^1-acetyltransferase (*SSAT*) has been definitively shown to have a polyamine responsive element in its upstream regulatory region *(13)*. Further, in the case of the transgenic mouse model, the overexpression of ODC in the keratinocytes was chronic in nature. These continuously elevated intracellular polyamine concentrations may have, over time, brought about a series of accommodative long-term changes in the metabolism or gene expression patterns in these keratinocytes that were responsible for the tumor promotion.

For these reasons, our laboratory became interested in the subset of genes whose levels of expression are affected in the short term by acute changes (either up or down) in intracellular polyamine pools. In an effort to identify "early" polyamine responsive genes, we developed model systems using the T-Rex system (Invitrogen) to produce a series of human cell lines containing an inducible antizyme-resistant truncated-ODC complimentary DNA. The resulting cell lines were named 293 ODC/Tet-Ind (derived from the 293 human embryonic kidney cell line); MCF-7 ODC/Tet-Ind (derived from the MCF-7 human breast cancer cell line); and LNCaP ODC/Tet-Ind (derived from the LNCaP human prostate cancer cell line). In the absence of tetracycline (TET), all of these cell lines had low basal levels of ODC activity and intracellular putrescine. When tetracycline was added to the culture media, ODC activities (and concomitant intracellular putrescine levels) in these cells could be rapidly induced to high levels in a dose-dependent manner *(14)*.

To identify the population of genes whose expression levels are altered by acute increases in intracellular putrescine concentrations, we treated the MCF-7 ODC/Tet-Ind and the 293 ODC/Tet-Ind cells with either TET alone, or in concert with exogenous substrate ornithine (ORN) or inhibitor DFMO. After 12 h (293) or 18 h (MCF-7), the cells were harvested and their polyamine contents determined. Total RNA from these cells were also collected. In both cell lines, the cells receiving TET or TET + ORN had significantly elevated intracellular polyamine levels. In contrast, the cells treated with DFMO and TET had intracellular polyamine levels that were identical to levels measured in control (untreated) cells. The RNA collected from the MCF-7 ODC/Tet-Ind and 293 ODC/Tet-Ind cells were subjected to genome-wide gene expression analysis using Affymetrix U95 human gene chip arrays.

On careful analysis of the resulting data from all treatments and cell types (data not shown), we identified a small number of messenger RNA (mRNA) whose expression

levels were significantly increased in response to elevated intracellular polyamines. Expected and found among these induced genes were *SSAT*, *c-FOS*, and *c-JUN*. However, we also found the dominant–negative transcription factor *ID-1* (inhibitor of differentiation-1) *(15–19)* and *RING3* (which reportedly interacts with E2F and modulates E2F-mediated transcription) *(20,21)* to be some of the polyamine-related genes.

In this chapter, we will detail some of the different ways in which polyamine pools can be manipulated to increase the net levels of expression of some of the mRNA, identified via genome-wide expression analysis in another ODC inducible cell line the LNCaP ODC/Tet-Ind cells. Our hypothesis was that the ability of elevated polyamines to increase the net accumulation of specific mRNA should not be cell type-specific.

1.1. A Stably Transfected TET-Inducible ODC System Effectively Increases Intracellular Polyamine Concentrations

To determine the extent of which TET alters intracellular polyamine levels after the induction of ODC in the cultured LNCaP ODC/Tet-Ind cells, the cell culture media was treated for up to 9 h with either 1 μg/mL exogenous TET, 1 μg/mL TET along with 1 m*M* DFMO, TET, and 1 m*M* ORN, 1 m*M* ORN only, or untreated (control). At 3-h intervals, cells were harvested, and their intracellular polyamine concentrations determined.

Figure 1A shows changes in intracellular putrescine. In the untreated cells, the intracellular putrescine levels were essentially unchanged at 6 nmol/mg protein over the 9-h time course. In contrast, the addition of TET caused a marked and steady increase in intracellular putrescine levels for the first 6 h to 160 nmol/mg protein followed by a slight decrease to 100 nmol/mg protein after 9 h. The addition of the ODC inhibitor, DFMO, effectively blocked the TET-mediated accumulation of intracellular putrescine. The addition of 1 m*M* ORN along with TET produced levels of intracellular putrescine similar to those seen in the TET only cells at 3 and 6 h. However, at 9 h, the TET + ORN cells had continued to accumulate intracellular putrescine concentrations to 250 nmol/mg protein. The addition of substrate ornithine alone in the absence of TET resulted in intracellular putrescine levels that were essentially unchanged from control concentrations.

Figure 1B illustrates the levels of intracellular spermidine over the 9-h time course. In the control, TET + DFMO and ORN-only treated cells exhibited intracellular spermidine levels that remained unchanged. However, in the cells receiving TET or TET + ORN, intracellular spermidine levels continued to decrease throughout the time course. At 9 h, the cells treated with TET + ORN had lost half of their intracellular spermidine, whereas the TET-only cells retained only 5% of the intracellular spermidine relative to the control values. Intracellular spermine values were relatively stable throughout the 9-h time course, with one notable exception being the TET-only cells, which lost 50% of the intracellular spermine between 6 and 9 h.

1.2. Similar Changes in Gene Expression Patterns Seen in Multiple Cell Types After Increases in ODC Activity

Would the genes identified from the genome-wide expression analysis of 293 ODC/Tet-Ind cells and MCF-7 ODC/Tet-Ind cells show altered gene expression in another cell line engineered to overexpress ODC? Using the same experimental treatments

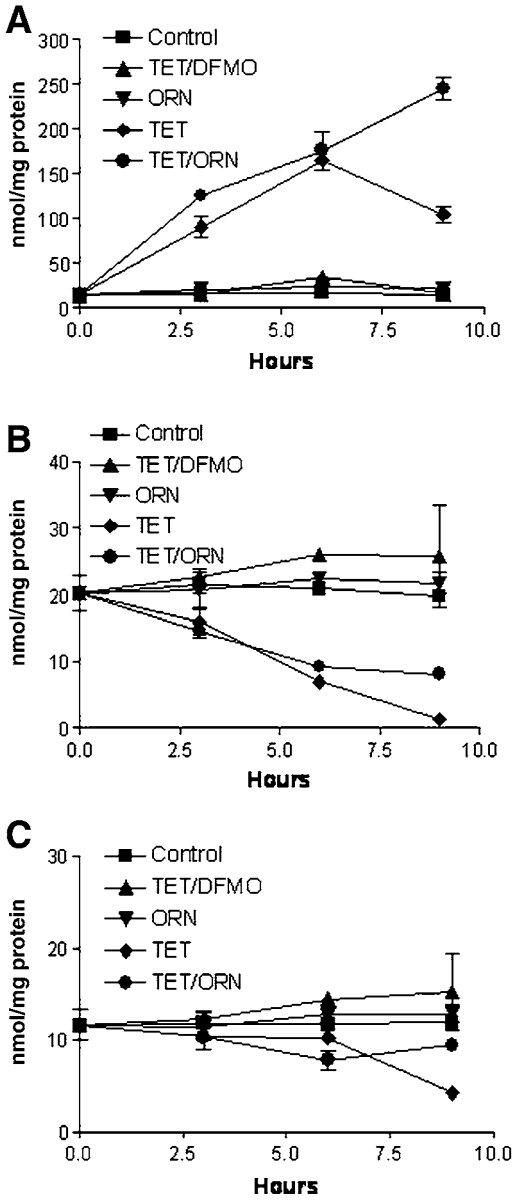

Fig. 1. Intracellular polyamine profile of LNCaP ornithine decarboxylase (ODC)/Tet-Ind cells after ODC induction by tetracycline for 3, 6, and 9 h. The cells were either untreated (control) or given 1 μg/mL tetracycline (TET), 1 μg/mL tetracycline and 1 m*M* α-difluoromethylornithine (DFMO) (TET/DFMO), 1 m*M* ornithine (ORN), or 1 μg/mL tetracycline and 1 m*M* ornithine (TET/ORN). The cell culture media was changed at time zero and contained the treatments indicated. To completely block the activity of ODC, the TET/DFMO cells were preincubated with 1 m*M* DFMO 1 h before the change of media. Values are means ± SEM of data repeated in triplicate.

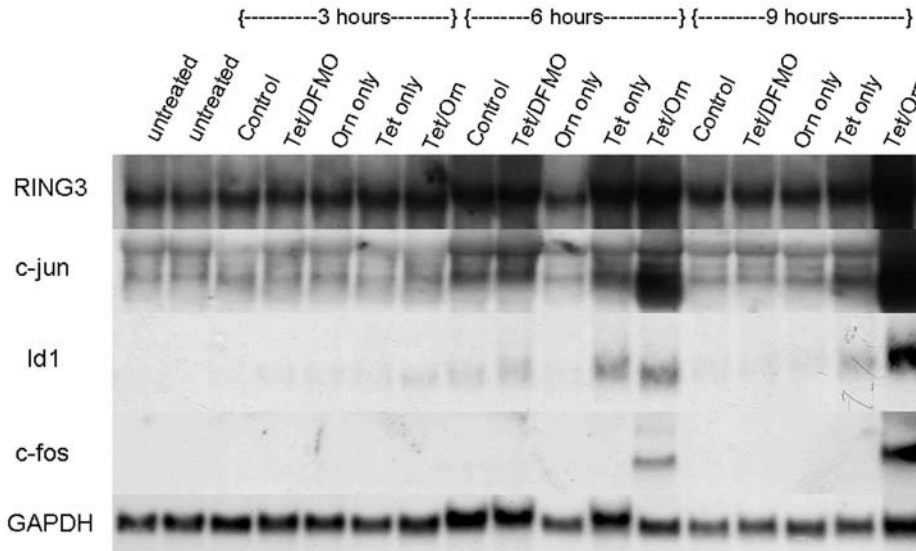

Fig. 2. Northern analysis of LNCaP ornithine decarboxylase (ODC)/Tet-Ind cells after ODC induction by tetracycline (TET) for 3, 6, and 9 h. The cells were either untreated (control) or given 1 µg/mL TET, 1 µg/mL tetracycline and 1 m*M* difluoromethylornithine (DFMO) (TET/DFMO), 1 m*M* ornithine (ORN), or 1 µg/mL tetracycline and 1 m*M* ornithine (TET/ORN). The cell culture media was changed at time zero and contained the treatments indicated. To completely block the activity of ODC, the TET/DFMO cells were preincubated with 1 m*M* DFMO 1 h before the change of media. Total RNA was isolated and subjected to Northern blot analysis with probes for *RING3*, *c-jun*, *Id-1*, and *c-fos*. Filters were then stripped and reprobed for *GAPDH*.

used previously, the LNCaP ODC/Tet-Ind cells were harvested at 3-h intervals and their steady-state mRNA levels of *RING3*, *c-jun*, *Id-1*, *c-fos*, and *GAPDH* determined. In the Northern blot pictured in Fig. 2, the control cells revealed no change in steady-state mRNA levels during the 9-h time course, whereas the TET-treated cells revealed increased steady-state mRNA levels of *RING3*, *c-jun*, and *Id-1* after 6 h and continued to remain elevated for the duration of the time course. The TET-mediated mRNA elevations of the genes were diminished through the addition of DFMO. The addition of ORN together with TET produced higher levels of steady-state mRNAs in *RING3*, *c-jun*, and *Id-1* genes than in the treatment with TET alone. The ORN + TET-treated cells also produced increased mRNA levels of the *c-fos* gene after 6 and 9 h. The cells treated with ORN alone resulted in no change from the control lanes.

1.3. Exogenous Administration of Putrescine Had No Effect of Cell Viability

To determine whether cell viability was affected by exogenous putrescine, we treated the LNCaP ODC/Tet-Ind cells with increasing exogenous putrescine concentrations for up to 72 h (Fig. 3). Even the cells treated with 30 m*M* exogenous putrescine had similar doubling times as control untreated LNCaP ODC/Tet-Ind cells for the initial

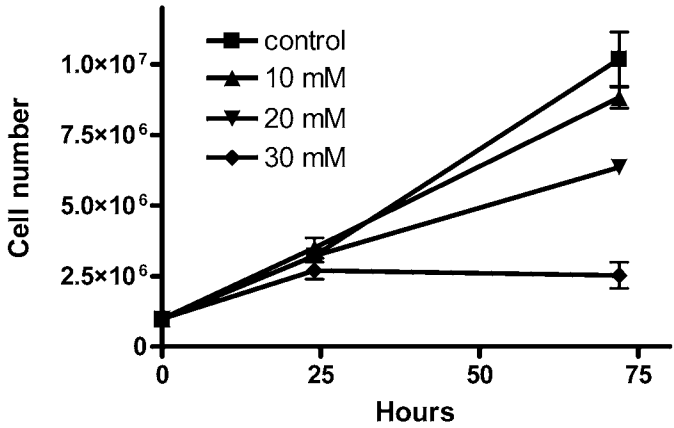

Fig. 3. Growth curve profile of LNCaP ornithine decarboxylase/Tet-Ind cells after the addition of 0, 10, 20, and 30 m*M* exogenous putrescine for a duration of 72 h. Trypan blue dye was added to a final concentration of 10%, and the viability of the cells was determined by counting blue stained (dead or dying) vs unstained (alive) cells on a hemacytometer. Results are a representative experiment that has been repeated in triplicate.

24 h. A dose-dependent reduction in the growth rate of the cells occurred after 72 h of incubation. However, cell viability remained at 100% after the initial doubling time of 24 h. The TET-mediated cells remained 100% viable throughout the 9-h time course.

1.4. Exogenous Putrescine Administration Capable of Inducing the Same Genes as Increased ODC Activity in a Time- and Dose-Dependent Manner

We wished to determine whether the addition of exogenous putrescine to the cultured media would alter intracellular polyamine concentrations in a similar manner as the TET-mediated ODC overexpression. Figure 4 shows that exogenous putrescine could alter intracellular polyamine concentrations in a dose-dependent manner when cultured LNCaP ODC/Tet-Ind cells were incubated with increasing concentrations of exogenous putrescine for 24 and 36 h. Similar changes in intracellular polyamine concentration occurred during the 24 and 36 h. Intracellular putrescine concentration increased eightfold because of increasing doses of exogenous putrescine (Fig. 4A). In a similar manner to treatments of TET + ORN, intracellular spermidine and spermine concentrations showed a decreasing trend as the exogenous putrescine concentrations increased (Fig. 4B,C). Intracellular spermidine and spermine decreased 90 and 80%, respectively, in cells treated with increasing exogenous putrescine.

Dose-dependent increases in steady-state mRNA levels of *RING3*, *Id-1*, and *c-jun* in response to increasing concentrations of exogenous putrescine after 24 and 36 h are shown in Fig. 5. At the times indicated, the cells were harvested and their total RNA isolated and subjected to Northern blot analysis. Similar changes in the levels of all three of the mRNA species were observed for each of the concentrations of exogenous putrescine after 24 and 36 h of exposure, with the possible exception of the 30 m*M*

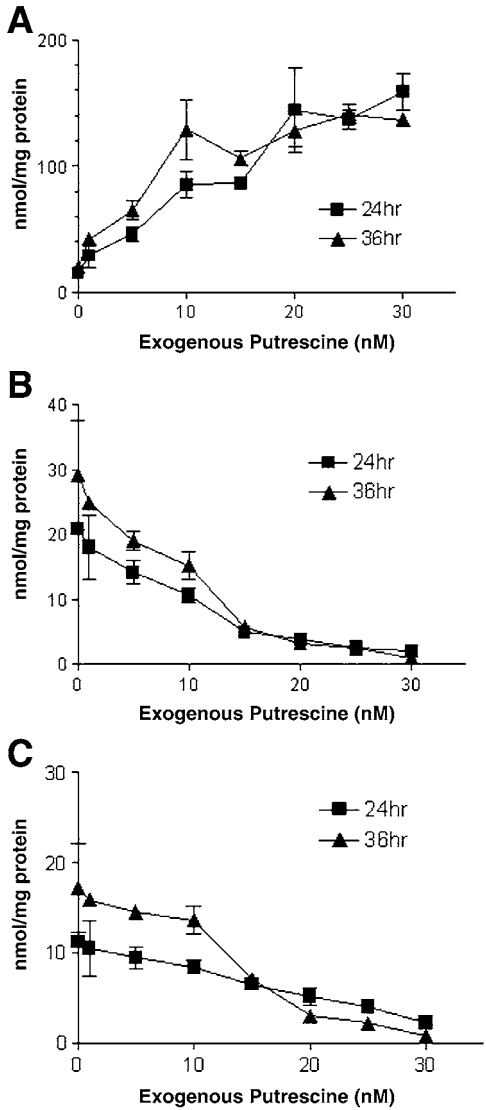

Fig. 4. Intracellular polyamine profile of LNCaP ornithine decarboxylase/Tet-Ind cells after the addition of either 1, 5, 10, 15, 20, 25, or 30 m*M* exogenous putrescine concentrations for 24 and 36 h. Putrescine was added directly to the media. After 24 or 36 h of incubation in putrescine at the indicated concentrations, the cells were harvested and their intracellular polyamine levels determined. Values are means ± SEM of data repeated in triplicate.

putrescine-exposed *c-jun*, which had 36-h mRNA levels that were slightly higher than those seen after 24 h. There was no significant increase over control levels in observed mRNA levels in the cells receiving 1 m*M* exogenous putrescine. However, incubation

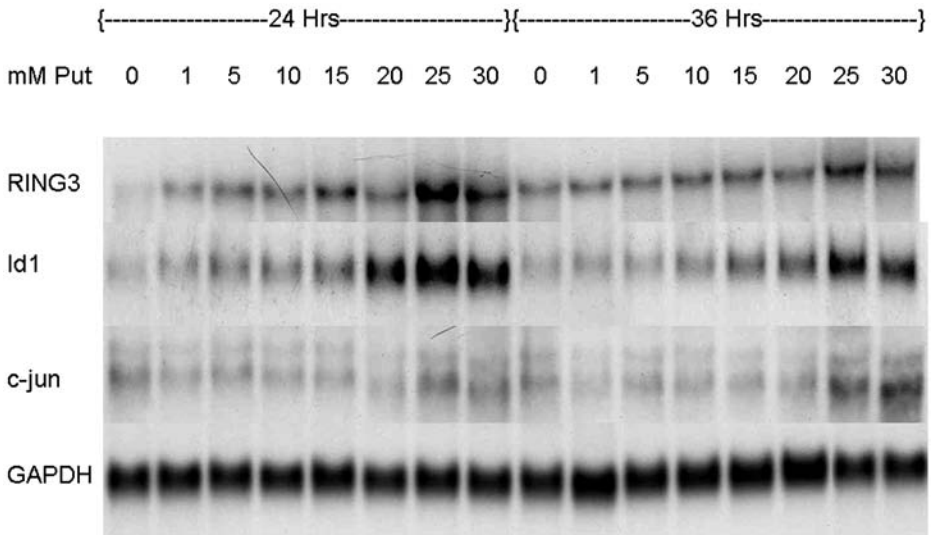

Fig. 5. Northern analysis of LNCaP ornithine decarboxylase/Tet-Ind cells after the addition of putrescine for 24 and 36 h. Putrescine was added directly to the media. After 24 or 36 h of incubation in putrescine at the indicated concentrations, total RNA was isolated and subjected to Northern blot analysis with probes for *RING3*, *c-jun*, *Id-1*, and *c-fos*. Filters were then stripped and reprobed for *GAPDH*.

with 5 m*M* exogenous putrescine did result in modest increases in the levels of *RING3*, *Id-1*, and *c-jun* mRNA. This indicates that the low end of the induction of these mRNAs was reached when exogenous putrescine levels were 5 m*M* or higher. This exogenous putrescine concentration resulted in intracellular putrescine levels of 50 nmol/mg protein, intracellular spermidine levels of 18 nmol/mg protein, and intracellular spermine levels of 10 nmol/mg protein (Fig. 4).

To ascertain the time-dependent changes in intracellular polyamine concentrations, the cells were treated with 30 m*M* exogenous putrescine for 24 h and harvested for polyamine analysis at 3, 6, 9, 12, and 24 h (Fig. 6). The intracellular putrescine concentration increased in a linear fashion for the initial 12 h, reaching a level approx 16-fold higher than measured in the control levels. The putrescine-treated cells also displayed a concomitant loss of spermidine and spermine during the 24 h incubation period. Intracellular spermidine and spermine concentrations decreased 88 and 70% after a delay of 3 and 9 h, respectively. Our cumulative data would indicate that intracellular spermidine and spermine levels do not decrease until intracellular putrescine concentrations reach approx 50 nmol/mg protein.

Cells treated with 30 m*M* exogenous putrescine were harvested for Northern blot analysis at 3, 6, 9, 12, and 24 h (Fig. 7). After 3 h of treatment, *Id-1* steady-state mRNA levels increased twofold from the control levels. The *Id-1* mRNA levels continued to increase throughout the 24-h time course, reaching a level of 15-fold higher than in control cells. *RING3* and *c-jun* mRNA levels also increased during the time course,

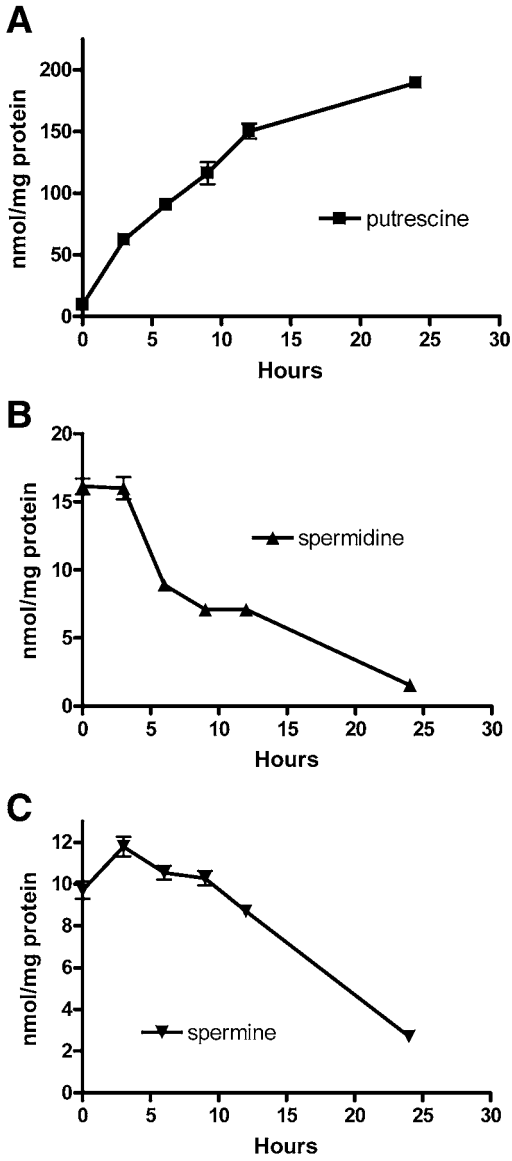

Fig. 6. Intracellular polyamine profile of LNCaP ornithine decarboxylase/Tet-Ind cells after the addition of 30 m*M* exogenous putrescine for 3, 6, 9, 12, and 24 h. Putrescine was added directly to the media. After incubation in putrescine for the times indicated, the cells were harvested and their intracellular polyamine levels determined. Values are means ± SEM of data repeated in triplicate.

although not to the same degree as observed with *Id-1*. In contrast, *c-fos* (unlike with TET-mediated ODC overexpression treatments) yielded no consistent change of mRNA levels with exogenous putrescine treatments. These results showed that the *Id-1*,

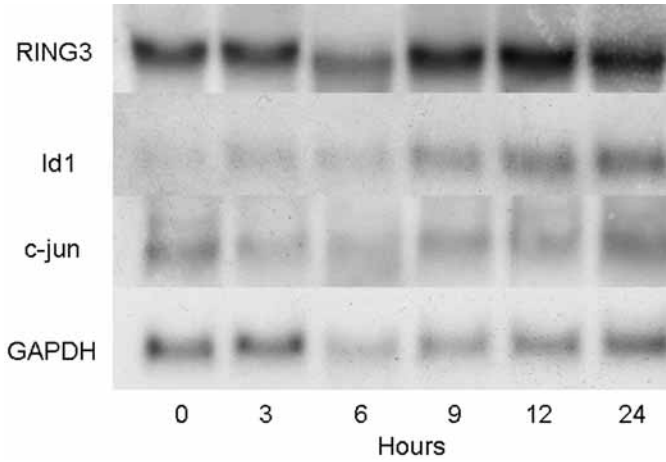

Fig. 7. Northern analysis of LNCaP ornithine decarboxylase/Tet-Ind cells after the addition of 30 m*M* exogenous putrescine for 3, 6, 9, 12, and 24 h. Putrescine was added directly to the media. After incubation in putrescine for times indicated, total RNA was isolated and subjected to Northern blot analysis with probes for *RING3*, *c-jun*, *Id-1*, and *c-fos*. Filters were then stripped and reprobed for *GAPDH*.

RING3, and *c-jun* genes increased in a time- and dose-dependent manner with exogenous putrescine. *Id-1* showed the most dramatic increases in steady-state mRNA levels, while *RING3* and *c-jun* were less affected. The *c-fos* mRNA levels were not visible after treatment with exogenous putrescine, but again ODC overexpression did lead to increases in *c-fos* (Fig. 2).

1.5. Increased SSAT Activity is Not Responsible for Induction of These Genes

The observed reduction in intracellular spermidine and spermine after treatment with exogenous putrescine or TET-mediated ODC overexpression might have been a result of increased SSAT activity. To determine the extent to which putrescine induces SSAT activity, the LNCaP ODC/Tet-Ind cells were grown for 24 h in media containing either 15 m*M* exogenous putrescine or 10 μ*M* DENSPM (N^1, N^{11}-diethylnorspermine, a spermine analog that induces high levels of SSAT activity) *(30)* (Table 1) before being harvested and assayed for SSAT activity. The cells receiving exogenous putrescine displayed an eightfold higher SSAT activity than the control cells (Table 1). The DENSPM-treated cells showed an 18-fold higher SSAT activity than the control cells. Our previous studies have shown that TET-mediated ODC overexpressing cells also raised SSAT activity 115-fold higher than control treated cells (data not shown).

ODC overexpression and exogenous putrescine raised intracellular putrescine levels, increased SSAT activity, and further caused a reduction in intracellular spermidine and spermine. We wished to determine whether it was the increase in intracellular putrescine or the reduction in intracellular spermidine or spermine that led to the increase in *RING3*, *Id-1*, and *c-jun* mRNA levels. Because DENSPM caused an increase in SSAT coupled with a reduction of intracellular putrescine (as well as spermidine and spermine), yet did

Table 1
SSAT Activity, Intracellular Polyamine Profile, and Relative Expression Levels of the Genes in LNCaP ODC/Tet-Ind Cells After Treatment of 15 m*M* Exogenous Putrescine or 10 μ*M* DENSPM for 24 h

	Control	Exogenous putrescine	DENSPM
SSAT activity (pmol [^{14}C]-acetylspermidine formed/mg protein × hour)	162.6 ± 3.2	1301.5 ± 142.7	2718.2 ± 218.6
Putrescine (nmol/mg protein)	15.2 ± 0.8	122.1 ± 8.5	11.9 ± 0.9
Spermidine (nmol/mg protein)	23.1 ± 0.9	7.7 ± 0.9	2.1 ± 0.1
Spermine (nmol/mg protein)	14.7 ± 0.4	9.9 ± 1.1	4.9 ± 0.4
RING3 (relative expression)	1	3.5	1.1
Id-1 (relative expression)	1	5.8	0.8
c-jun (relative expression)	1	2.2	1
c-fos (relative expression)	1	0.9	0.9

not alter the specific mRNA levels (Table 1), we concluded that increased intracellular putrescine was mediating the alteration of the mRNA levels.

To study the early changes in patterns of gene expression after acute increases in intracellular putrescine concentration, we performed Affymetrix gene chip analysis on mRNAs from human 293 and MCF-7 cell lines containing a stably transfected TET-inducible ODC construct. Among the genes identified as upregulated after induction of ODC in the presence of substrate ORN were *c-fos*, *c-jun*, *RING3*, and *Id-1*. Using another human cell line (LNCaP) containing this inducible ODC construct to confirm the previous observations, we analyzed the changes in the patterns of expression of these genes after alterations in intracellular polyamine pools mediated either by overexpression of ODC or administration of exogenous putrescine. Large increases in intracellular putrescine, in addition to inducing these genes, were accompanied by decreases in intracellular spermidine and spermine. Studies with the inducer of SSAT, DENSPM, mirrored the decreases in intracellular spermidine and spermine without the increase in intracellular putrescine or induction of the genes. Our data indicate that the threshold of induction for these mRNAs is time- and dose-dependent and that, in the short term, intracellular putrescine levels of at least 50 nmol/mg proteins for a period longer than 3 h were capable of significantly increasing the steady-state levels of the mRNAs coding for these growth or cancer-related genes.

2. Possible Mechanisms and Conclusion

Several mechanisms of polyamine-mediated induction of gene expression have been proposed. Some studies have suggested a mechanism involving changes in DNA structure and conformation either through histone acetylation or by enhancing the interaction between nuclear transcription factors and steroid hormone receptors *(22–25)*.

A polyamine-responsive element (PRE) upstream of the transcription start site of SSAT has been identified as another mechanism of how polyamines may modulate

transcription *(13)*. NF-E2-related transcription factor 2 is a nuclear factor that has been reported to bind to the PRE of the *SSAT* gene. Two other proposed ligands of the PRE are the mammalian homolog of the *Arabidopsis* COP 9 signalosome subunit 7a and the polyamine modulated factor 1 *(26–28)*. The exact mechanisms by which these ligands modulate the transcription of SSAT have yet to be determined.

Data from other investigators demonstrating changes in specific gene expression after polyamine depletion with DFMO suggest that intracellular polyamines regulate the expression of specific genes *(29–37)*. To lower polyamine levels, the most often used approach was to use DFMO to deplete intracellular putrescine, which would then result in decreased intracellular polyamine pools, mRNA, and the resulting protein product of the polyamine-responsive genes. Additionally, this DFMO effect was ablated by the simultaneous administration of exogenous polyamines *(30–37,38)*. This depletion model has revealed decreases in steady-state expression levels of the RNA coding for *c-myc*, *c-fos*, *c-jun*, *4E-BP1*, *SSAT*, and *IκBα* in a number of different cell lines *(30–37,39)*.

Our data show that increases in intracellular putrescine lead to net changes in levels of gene expression. Two distinct methods were used to raise intracellular putrescine concentrations in LNCaP ODC/Tet-Ind cells and increase the steady-state levels of *RING3*, *Id-1*, *c-jun*, and *c-fos* mRNAs. Either TET-mediated ODC overexpression or the administration of exogenous putrescine were capable of producing sustained increases in intracellular putrescine and elevated steady-state mRNA levels. Although we have yet to identify the mechanisms by which these mRNAs are induced by polyamines, we are in the process of analyzing the upstream regions of these genes for elements capable of activating reporter gene transcription under conditions of elevated intracellular polyamine levels.

It is interesting to note that the genes identified as upregulated by our microarray analysis of cells with elevated intracellular putrescine had been previously shown to be involved either in growth or cancer. AP-1 factors c-jun and c-fos have been well established in their involvement in tumorigenesis *(40)*. In addition to transcriptional and translational regulation appearing to stem from the *ras* signaling pathway *(40)*, other investigators have demonstrated that exogenous polyamines are capable of causing increases in *c-fos* and *c-myc* gene expression *(37)*.

RING3 (really interesting new gene 3) (recently renamed BRD2), is a serine-threonine kinase that participates in nuclear protein complexes associated with E2F *(20)*. *RING3* has been shown to transactivate E2F-dependent cell-cycle regulatory genes. In concert with activated *ras*, *RING3* was also shown to induce the transformation of NIH/3T3 cells. It has also been speculated that activated *ras* may mediate the phosphorylation of inactive *RING3 (21)*.

Id-1 (inhibition of differentiation) belongs to the Id family of proteins, which are known to bind with basic helix–loop–helix transcription factors and prevent them from binding DNA. The net effect of *Id-1* binding is a dominant negative regulation of the bound transcription factor and the silencing of the associated genes. These target genes are generally associated with lineage-specific expression and differentiation *(41)*. *Id-1* gene expression has been shown to be elevated in a number of primary tumors vs

normal control tissue specimens *(40)*. In a review by Norton and colleagues *(41)*, *Id-1* regulation was found to be regulated by the expression of immediate early gene *EGR-1*; however, Id-1 protein is involved in a negative feedback loop that downregulates the formation of *ECR-1* and *c-fos*. This may explain the decrease in expression of *c-fos* mRNA we observed at later time points when the expression of *Id-1* was highest.

At first, we were unsure whether the changes in gene expression observed were the result of the increased intracellular putrescine stemming from the TET-mediated overexpression of ODC or the concomitant decreases in the other polyamines. The data in Table 1 strongly suggest that the decreases in intracellular spermine and spermidine observed after ODC induction did not play a substantial role in the inductions of *RING3*, *Id-1*, *c-jun*, or *c-fos*.

Our data indicate that only when intracellular putrescine levels were significantly increased could elevated steady-state mRNA levels of *RING3*, *Id-1*, *c-jun*, and *c-fos* be measured. The induction of these mRNAs appears to occur when intracellular putrescine concentrations greater than 50 nmol/mg protein are sustained for three or more hours. A further refinement of the putrescine levels and times of exposure that are necessary for the induction of these genes is under way in our laboratory.

Acknowledgments

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References

1. Pegg, A. E., Feith, D. J., Fong, L. Y., Coleman, C. S., O'Brien, T. G., and Shantz, L. M. (2003) Transgenic mouse models for studies of the role of polyamines in normal, hypertrophic and neoplastic growth. *Biochem. Soc. Trans.* **31,** 356–360.
2. Koza, R. A., Megosh, L. C., Palmieri, M., and O'Brien, T. G. (1991) Constitutively elevated levels of ornithine and polyamines in mouse epidermal papillomas. *Carcinogenesis* **12,** 1619–1625.
3. Shantz, L. M., Guo, Y., Sawicki, J. A., Pegg, A. E., and O'Brien, T. G. (2002) Overexpression of a dominant-negative ornithine decarboxylase in mouse skin: effect on enzyme activity and papilloma formation. *Carcinogenesis* **23,** 657–664.
4. Ahmad, N., Gilliam, A. C., Katiyar, S. K., O'Brien, T. G., and Mukhtar, H. (2001) A definitive role of ornithine decarboxylase in photocarcinogenesis. *Am. J. Pathol.* **159,** 885–892.
5. Gilmour, S. K., Robertson, F. M., Megosh, L., O'Connell, S. M., Mitchell, J., and O'Brien, T. G. (1992) Induction of ornithine decarboxylase in specific subpopulations of murine epidermal cells following multiple exposures to 12-O-tetradecanoylphorbol-13-acetate, mezerein and ethyl phenylpropriolate. *Carcinogenesis* **13,** 51–56.
6. Megosh, L., Gilmour, S. K., Rosson, D., et al. (1995) Increased frequency of spontaneous skin tumors in transgenic mice which overexpress ornithine decarboxylase. *Cancer Res.* **55,** 4205–4209.
7. Guo, Y., Zhao, J., Sawicki, J., Peralta, S. A., and O'Brien, T. G. (1999) Conversion of C57Bl/6 mice from a tumor promotion-resistant to a -sensitive phenotype by enhanced ornithine decarboxylase expression. *Mol. Carcinog.* **26,** 32–36.

8. Boutwell, R. K. (1964) Some biological aspects of skin carcinogenesis. *Prog. Exp. Tumor Res.* **19,** 207–250.
9. Boutwell, R. K. (1974) The function and mechanism of promoters of carcinogenesis. *CRC Crit. Rev. Toxicol.* **2,** 419–443.
10. Slaga, T. J. (1989) Cellular and molecular mechanisms involved in multistage skin carcinogenesis. *Carcinog. Compr. Surv.* **11,** 1–18.
11. Smith, M. K., Trempus, C. S., and Gilmour, S. K. (1998) Co-operation between follicular ornithine decarboxylase and v-Ha-ras induces spontaneous papillomas and malignant conversion in transgenic skin. *Carcinogenesis* **19,** 1409–1415.
12. Lan, L., Trempus, C., and Gilmour, S. K. (2000) Inhibition of ornithine decarboxylase (ODC) decreases tumor vascularization and reverses spontaneous tumors in ODC/Ras transgenic mice. *Cancer Res.* **60,** 5696–5703.
13. Wang, Y., Xiao, L., Thiagalingam, A., Nelkin, B. D., and Casero, R. A., Jr. (1998) The identification of a cis-element and a trans-acting factor involved in the response to polyamines and polyamine analogues in the regulation of the human spermidine/spermine N1-acetyltransferase gene transcription. *J. Biol. Chem.* **273,** 34,623–34,630.
14. Wilson, S. M., Hawel, L., III, Pastorian, K. E., and Byus, C. V. (2005) A stable, inducible, dose-responsive ODC overexpression system in three human cell lines. *Biochimica et Biophysical Acta* **1732,** 103–110.
15. Desprez, P. Y., Hara, E., Bissell, M. J., and Campisi, J. (1995) Suppression of mammary epithelial cell differentiation by the helix-loop-helix protein Id-1. *Mol. Cell Biol.* **15,** 3398–3404.
16. Desprez, P. Y., Lin, C. Q., Thomasset, N., Sympson, C. J., Bissell, M. J., and Campisi, J. (1998) A novel pathway for mammary epithelial cell invasion induced by the helix-loop-helix protein Id-1. *Mol. Cell. Biol.* **18,** 4577–4588.
17. Singh, J., Itahana, Y., Parrinello, S., Murata, K., and Desprez, P. Y. (2001) Molecular cloning and characterization of a zinc finger protein involved in Id-1-stimulated mammary epithelial cell growth. *J. Biol. Chem.* **276,** 11,852–11,858.
18. Fong, S., Debs, R. J., and Desprez, P. Y. (2004) Id genes and proteins as promising targets in cancer therapy. *Trends Mol. Med.* **10,** 387–392.
19. Coppe, J. P., Itahana, Y., Moore, D. H., Bennington, J. L., and Desprez, P. Y. (2004) Id-1 and Id-2 proteins as molecular markers for human prostate cancer progression. *Clin. Cancer Res.* **10,** 2044–2051.
20. Guo, N., Faller, D. V., and Denis, G. V. (2000) Activation-induced nuclear translocation of RING3. *J. Cell Sci.* **113,** 3085–3091.
21. Denis, G. V., Vaziri, C., Guo, N., and Faller, D. V. (2000) RING3 kinase transactivates promoters of cell cycle regulatory genes through E2F. *Cell. Growth Differ.* **11,** 417–424.
22. Hobbs, C. A., and Gilmour, S. K. (2000) High levels of intracellular polyamines promote histone acetyltransferase activity resulting in chromatin hyperacetylation. *J. Cell Biochem.* **77,** 345–360.
23. Hobbs, C. A., Paul, B. A., and Gilmour, S. K. (2002) Deregulation of polyamine biosynthesis alters intrinsic histone acetyltransferase and deacetylase activities in murine skin and tumors. *Cancer Res.* **62,** 67–74.
24. Hobbs, C. A., Paul, B. A., and Gilmour, S. K. (2003) Elevated levels of polyamines alter chromatin in murine skin and tumors without global changes in nucleosome acetylation. *Exp. Cell. Res.* **290,** 427–436.
25. Maeda, Y., Rachez, C., Hawel, L., III, Byus, C. V., Freedman, L. P., and Sladek, F. M. (2002) Polyamines modulate the interaction between nuclear receptors and vitamin D receptor-interacting protein 205. *Mol. Endocrinol.* **16,** 1502–1510.

26. Wang, Y., Devereux, W., Stewart, T. M., and Casero, R. A., Jr. (2001) Characterization of the interaction between the transcription factors human polyamine modulated factor (PMF-1) and NF-E2-related factor 2 (Nrf-2) in the transcriptional regulation of the spermidine/spermine N1-acetyltransferase (SSAT) gene. *Biochem. J.* **355,** 45–49.
27. Wang, Y., Devereux, W., Woster, P. M., Stewart, T. M., Hacker, A., and Casero, R. A., Jr. (2001) Cloning and characterization of a human polyamine oxidase that is inducible by polyamine analogue exposure. *Cancer Res.* **61,** 5370–5373.
28. Wang, Y., Devereux, W., Woster, P. M., and Casero, R. A., Jr. (2001) Cloning and characterization of the mouse polyamine-modulated factor-1 (mPMF-1) gene: an alternatively spliced homologue of the human transcription factor. *Biochem. J.* **359,** 387–392.
29. Celano, P., Baylin, S. B., Giardiello, F. M., Nelkin, B. D., and Casero, R. A., Jr. (1988) Effect of polyamine depletion on c-myc expression in human colon carcinoma cells. *J. Biol. Chem.* **263,** 5491–5494.
30. Celano, P., Baylin, S. B., and Casero, R. A., Jr. (1989) Polyamines differentially modulate the transcription of growth-associated genes in human colon carcinoma cells. *J. Biol. Chem.* **264,** 8922–8927.
31. Wang, J. Y., McCormack, S. A., Viar, M. J., et al. (1993) Decreased expression of protooncogenes c-fos, c-myc, and c-jun following polyamine depletion in IEC-6 cells. *Am. J. Physiol.* **265,** G331–G338.
32. Liu, L., Li, L., Rao, J. N., et al. (2005) Polyamine-modulated expression of c-myc plays a critical role in stimulation of normal intestinal epithelial cell proliferation. *Am. J. Physiol. Cell Physiol.* **288,** C89–C99.
33. Li, L., Rao, J. N., Bass, B. L., and Wang, J. Y. (2001) NF-kappaB activation and susceptibility to apoptosis after polyamine depletion in intestinal epithelial cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* **280,** G992–G1004.
34. Stephenson, A. H., Christian, J. F., and Seidel, E. R. (2004) Polyamines regulate eukaryotic initiation factor 4E-binding protein 1 gene transcription. *Biochem. Biophys. Res. Commun.* **323,** 204–212.
35. Kramer, D. L., Khomutov, R. M., Bukin, Y. V., Khomutov, A. R., and Porter, C. W. (1989) Cellular characterization of a new irreversible inhibitor of S-adenosylmethionine decarboxylase and its use in determining the relative abilities of individual polyamines to sustain growth and viability of L1210 cells. *Biochem. J.* **259,** 325–331.
36. Patel, A. R. and Wang, J. Y. (1997) Polyamines modulate transcription but not posttranscription of c-myc and c-jun in IEC-6 cells. *Am. J. Physiol.* **273,** C1020–C1029.
37. Tabib, A. and Bachrach, U. (1994) Activation of the proto-oncogene c-myc and c-fos by c-ras: involvement of polyamines. *Biochem. Biophys. Res. Commun.* **202,** 720–727.
38. Celano, P. Baylin, S. B., Giardiello, F. M., Nelkin, B. D., and Casero, R. A., Jr. (1988) Effect of polyamine depletion on c-myc expression in human colon carcinoma cells. *J. Biol. Chem.* **263,** 5491–5494.
39. Eferl, R. and Wagner, E. F. (2003) AP-1: a double-edged sword in tumorigenesis. *Nat. Rev. Cancer* **3,** 859–868.
40. Sikder, H. A., Devlin, M. K., Dunlap, S., Ryu, B., and Alani, R. M. (2003) Id proteins in cell growth and tumorigenesis. *Cancer Cell* **3,** 525–530.
41. Norton, J. D. (2000) ID helix-loop-helix proteins in cell growth, differentiation and tumorigenesis. *J. Cell Sci.* **113,** 3897–3905.

3

Polyamine-Dependent Early Cellular Signals and Cell Proliferation

Stina M. Oredsson

1. Introduction

When a resting cell is awakened to enter the cell cycle by mitogenic stimulation, a complex molecular machinery involving positive and negative regulation of cell-cycle progression is initiated. Part of the machinery is quite well-known. It involves receptors of various kinds, protein interactions, phosphorylation reactions, and molecular cascades carrying the signal to enter the cell cycle from the cell surface to the nucleus. In the nucleus, transcriptional activation results in an increased involvement of genes in the process. In the cytoplasm, translational and posttranslational mechanisms also have a part in the process. In this complex partly known molecular machinery, there are also molecules that not yet have been assigned a specific role; however, there are enough data to state that their role in normal cell-cycle progression is ubiquitous. The level of the polyamines—putrescine, spermidine, and spermine—are low in nonproliferating cells, but their levels increase early after mitogenic stimulation and these increases are necessary for normal rates of cell-cycle progression *(1,2)*. In this chapter, the focus will be on the role of polyamines in early signaling processes that are required for normal cell-cycle progression.

2. Mitogenically Activated Signal Transduction Pathways

Signal transduction is the process by which information from an extracellular signal is transmitted from the plasma membrane into the cell and along an intracellular chain of signaling molecules to stimulate a cellular response. Mitogenic stimulation, when a growth factor binds to its receptor on the cell surface, is such a process. This stimulation initiates a number of parallel signal transduction pathways, the combined role of which is to achieve a balanced regulation of the initiated signal.

Mitogenic stimuli initiate cell proliferation via different classes of cell surface receptors, which include growth factor receptor tyrosine kinases and G protein-coupled receptors. This results in tyrosine phosphorylation of Shc and the sequential activation

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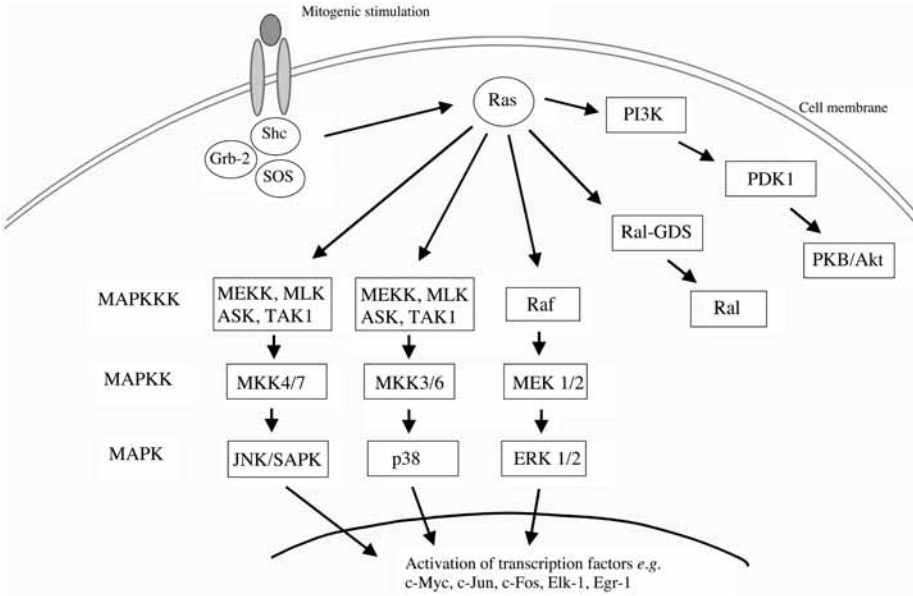

Fig. 1. Schematic representation of Ras-activated signal transduction pathways. The three-tiered MAPK pathways JNK/SAPK, p38, and Erk 1/2 are shown where MAPKKK, MAPKK, and MAPK show the first, second, and third phosphorylation levels, respectively. Ras also activates phosphatidylinositol 3-kinase (PI3K)-mediated and Ral guanine-nucleotide-exchange factor (GDS)-mediated signals (Ral-GDS). Ras denotes a superfamily of small GTPases.

of Grb2, SOS, and then Ras *(3,4)* (Fig. 1). Ras activity is controlled by a regulated guanosine 5′-diphosphate/guanosine 5′-triphosphate cycle where only guanosine 5′-triphosphate-bound Ras is active. The Shc/Grb2/SOS complex stimulates the formation of active guanosine 5′-triphosphate-Ras from inactive guanosine 5′-diphosphate-Ras. Here Ras denotes a superfamily of small guanosine 5′-triphosphatases of more than 80 mammalian members. The prototypic Ras proteins are H-Ras, K-Ras, and N-Ras. Ras activation then results in the activation of the ERK1/2 (p42/44) mitogen-activated protein kinase (MAPK pathway) through Raf. Ras-mediated activation of Raf is a complex, multistep process *(3)*. Phosphorylated ERK1 (p44) and 2 (p42) then enter the cell nucleus and activate a variety of substrates that include the Elk-1, Egr-1, c-Jun, and c-Myc transcription factors. One of the transcriptional targets of c-Myc is ornithine decarboxylase (ODC), the initial enzymatic step in polyamine biosynthesis *(5)*. Thus the major role of the ERK1/2 pathway is transcriptional activation. The ERK1/2 pathway is the classical three-tiered MAPK pathway where kinase phosphorylation takes place at three levels (Fig. 1).

In addition to activation of the ERK1/2 MAPK pathway, Ras activation also initiates a number of other Raf-independent signal transduction pathways *(3,6)*. Such pathways are phosphatidylinositol 3-kinase (PI3K)-mediated and Ral guanine-nucleotide-exchange factor (GDS)-mediated signals. Ras binds to and activates the catalytic sub-

unit of PI3K, a lipid kinase. This results in the formation of phosphatidylinositol 3,4,5-triphosphate, a second messenger, that, via the phosphoinositide-dependent kinase 1, results in the activation of the protein kinase B/Akt pathway. Protein kinase B/Akt phosphorylates many proteins involved in cell-cycle progression *(7,8)*. One of the master regulators of translational control is the target of rapamycin (TOR) protein, which controls the translational apparatus through protein phosphorylation *(6)*. The key regulators of translation that are controlled by TOR include the p70 ribosomal S6 kinases (S6K1 and S6K2) and 4E-BP1. The TOR-S6K pathway is regulated by signals that are transmitted in response to mitogenic stimulation through PI3K. Phosphatidylinositol 3,4,5-triphosphate also contributes to the activation of the ERK1/2 pathway *(6)*. Ral-GDS activates Ral, which has a role in both transcriptional and translational activation of cell-cycle regulatory proteins *(6)*.

Besides the classic ERK1/2 MAPK cascade, there are two other three-tiered MAPK pathways, the C-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) MAPK and the p38 MAPK pathways (Fig. 1). These are also not only activated by growth factors, but also by other kinds of stimuli such as ultraviolet-, heat shock-, and high osmosis-induced stress *(3,9)*. Ras activation is also important for the JNK/SAPK and p38 MAPK pathways; however, they are Raf-independent pathways as are the PI3K and Ral-DGS pathways mentioned previously. The p38 MAPK pathway has been implicated as an important negative regulator of cell-cycle progression *(10)*. Activation of the p38 MAPK pathway results in regulation of both transcription and translation of a number of genes involved in the negative control of cell-cycle progression, but also in the stimulation of proteasome-dependent degradation of proteins that stimulate cell-cycle progression. A major target of the JNK/SAPK signaling pathway is the activation of the AP-1 transcription factor that in part is mediated by the phosphorylation of c-Jun *(11)*. The three-tiered kinase ladders of the JNK/SAPK and p38 MAPK pathways involve more kinases than the ERK1/2 MAPK pathway *(9)*.

In view of its very central role in signal transduction, it is not surprising that Ras family proteins are oncogenes belonging to a family of structurally similar oncogenic proteins *(6,12)*. Closely related to the Ras family proteins are the Rho family proteins *(12)*. The Rho family proteins are regulators of actin organization, gene expression, and cell-cycle progression and have been implicated to have a role in Ras-mediated transformation in the JNK/SAPK and p38 MAPK pathways. Ras initiates the cell cycle through MAPK pathways and other pathways, but subsequent action of the Rho family is required for completion of the process.

Characteristic for these pathways is that they do not function on their own. There is extensive cross talk making it difficult to study the role of a single pathway in cell-cycle regulation. In addition, there are other signal transduction pathways, such as the cytokine-stimulated pathways and the transforming growth factor (TGF)-β pathway *(13,14)*. The specific cellular response is the sum of the total contribution of the individual pathways. Is there a place for the polyamines in this complex pattern making it even more complex? There are several studies of the role of polyamines in signal transduction that implicate that they have a function. The studies are of two major kinds. In many of the early studies, cells were depleted of their polyamines and the downstream

appearance of transcription factors was studied. The transcription factors studied were c-Jun, c-Fos, Egr-1, and c-Myc, all dependent on the described pathways. In later studies, the specific activation of the MAPK pathways and other pathways has been studied in relation to activation of polyamine biosynthesis.

3. Polyamine Depletion and the Expression of Transcription Factors

In many of the early articles pointing to a role of polyamines in signal transduction, the cells were depleted of their polyamines by treatment with α-difluoromethylornithine (DFMO) *(15)*. DFMO is an enzyme-activated irreversible inhibitor of ODC. DFMO treatment results in the depletion of putrescine and spermidine, whereas spermine essentially remains unchanged.

A significant decrease in the steady-state levels of one or more of the transcription factors *c-myc*, *c-fos*, and *c-jun* messenger RNA (mRNA) was found in the human colon carcinoma cell line COLO320 *(16)* and in the rat intestinal epithelial cell line IEC-6 *(17,18)* after DFMO treatment. There was not a decrease in general mRNA expression pointing to a specific role for polyamines regarding *c-myc* and *c-jun* mRNA expression. Nuclear run-on assays showed that the basal rates of transcription of *c-fos* and *c-myc* were decreased in polyamine-depleted nuclei from IEC-6 cells *(18,19)*. However, there was no difference in the stability ($t_{1/2}$) of the *c-myc* and *c-jun* messages in control and polyamine-depleted cells *(18)*. When DFMO-treated cells were stimulated with serum, there was no increase in the mRNA levels of the observed transcription factors *(17,18)*. Spermidine supplementation to DFMO-treated IEC-6 cells restored *c-myc* and *c-jun* mRNA levels. It was suggested that regulation of transcription factor expression may be one of the mechanisms by which polyamines modulate cell growth.

In a model using rat kidney cells infected with a temperature-sensitive mutant of Kirsten sarcoma virus, it was shown that polyamines stimulate transcription of *c-myc* and *c-fos* and the expression was blocked by DFMO treatment *(20)*. In another study, it was shown that DFMO-induced depletion of putrescine and spermidine in SV40-transformed 3T3 cells prevented *c-jun* mRNA induction by insulin; however, it did not affect *c-jun* mRNA induction by vanadate *(21)*. Insulin and vanadate function through different signal transduction pathways: insulin requires G protein, whereas vanadate does not. Spermidine addition to the DFMO-treated cells restored the stimulating effect of insulin. Thus polyamines appeared to be required for the G protein-mediated insulin-stimulated signal but not for the vanadate-stimulated signal.

Schulze-Lohoff and colleagues *(22)* grew rat renal mesangial cells in the presence of DFMO for 4 d and then deprived the cells of fetal calf serum (FCS) for 2 d. The cells were then stimulated with FCS, and the mRNA levels of *c-fos*, *c-jun*, and *Egr-1* were investigated. The researchers found that the expression of the genes did not increase to the same extent in polyamine-depleted cells as in control cells.

All these studies implicate a role for the polyamines in the efficient expression of the transcription factors *c-fos*, *c-jun*, *c-myc*, and *Egr-1*—all being effector molecules of MAPK signal transduction activation. In contrast to the studies mentioned, Charollais and Mester *(23)* found that the *c-myc* expression was not inhibited by polyamine deple-

tion and serum starvation in BALB/c-3T3 fibroblasts. In fact, both *c-myc* and *ODC* were expressed at relatively high levels in the serum-starved, polyamine-depleted cells. These cells could be stimulated to grow with nondialyzed FCS but not with dialyzed FCS that did not contain polyamines. However, a combination of epidermal growth factor, insulin, and putrescine resulted in immediate stimulation. These results imply that during 3 d of DFMO treatment and 3 d of serum starvation, the polyamine levels were depleted to such low levels that serum stimulation without putrescine had no effect (i.e., the growth-stimulating signals through various receptors were not functional despite the increased *c-myc* and *ODC* mRNA levels). In similarity with Charollais and Mester *(23)*, we found that *c-fos*, *c-jun*, ODC, and *AdoMetDC* mRNA levels were higher in polyamine-depleted and serum-starved Chinese hamster ovary cells than in control cells (unpublished results, K. Alm and S. Oredsson). That polyamine depletion resulted in different effects on mRNA levels of transcription factors and polyamine biosynthetic enzymes may be a result of the different genetic lesions in the cell lines, with the tumor suppressor p53 being a possible candidate. It has been shown that cells with wild-type p53 appear to be blocked in G_1 by polyamine depletion, whereas cells with mutated p53 do not show any specific cell-cycle block *(24,25)*.

Bauer and colleagues *(26)* showed that inhibition of ODC and putrescine production by DFMO in rat aortic smooth muscle cells resulted in activation of the ERK1/2 MAPK pathway to promote induction of p21 and consequently inhibition of cell proliferation. Activation of ERK1/2 MAPK and induction of p21was inhibited by putrescine addition.

4. Signal Transduction Pathways and Activation of Polyamine Biosynthesis

It was noted early that ODC activity was rapidly and dramatically elevated by a variety of exogenous stimuli that were coupled to several signal transduction pathways *(27–29)*.

The involvement of the Raf/MEK/ERK and PI3K pathways in ODC induction was shown in DFMO-resistant HL-60 cells that were stimulated to grow from quiescence *(30,31)*. When ERK1/2 phosphorylation was inhibited, there was a reduction in the accumulation of *ODC* mRNA and protein; however, ODC protein turnover was hardly affected *(31)*. Also, the involvement of Src, Ras, PI3K, and G-coupled proteins in ODC induction was shown using different inhibitors. When serum-starved human ECV304 cells were treated with histamine or adenosine triphosphate, there was a transient induction of ODC *(32)*. The agents also provoked an increase in active phosphorylated ERK1/2 and p38 MAPKs. Specific inhibition of ERK1/2 MAPK prevented the induction of ODC, whereas specific inhibitors of p38 MAPK enhanced induction of ODC in a way that appeared dependent on ERK1/2 MAPK. It has been reported that PD98059-mediated inhibition of ERK1/2 MAPK phosphorylation prevented the expression of *c-myc* and *c-fos (33)* and the ODC activity *(31)*. Addition of polyamines to NIH 3T3 cells reversed PD98059-mediated inhibition on MAPK phosphorylation and expression of *c-myc* and *c-fos (33)*. In serum-starved NIH 3T3 fibroblasts, spermidine preferentially stimulated transcription and translation of *c-myc*, whereas putrescine stimulated transcription and translation of *c-fos (34,35)*.

Several studies have shown that the ODC gene is deregulated in *ras*-transformed cells *(36–40)*. ODC overproducing transfectants showed enhanced MAPK *(31)* and tyrosine kinase activities *(41)*. MAPK activation has also been found in response to polyamine excess *(42,43)*.

NIH 3T3 cells transfected with c-Ha-*ras* showed markedly enhanced ODC activity and ODC protein level *(36)*. An increased *ODC* mRNA level as a result of an increased rate of transcription could account for this observation. The turnover rate of *ODC* mRNA was also decreased in transformed cells and the ODC protein was stabilized. The results suggested ODC deregulation at multiple levels in *ras*-oncogene-transformed cells. Similar results have been found for AdoMetDC and for ODC in H-Ras-transformed mouse 10T1/2 cells *(44)*. Experiments using NIH 3T3 cells transfected with plasmids encoding activated mutants for H-Ras or RhoA suggested that the increase in ODC activity at least partly was through a Raf/MEK/ERK-independent pathway *(39)*. To further delineate the role of different Ras effector pathways in ODC activation following stable *ras* transfection, NIH 3T3 cells were transfected with partial loss-of-function Ras mutants to selectively activate pathways downstream of Ras *(40)*. Mutants that selectively activate signal transduction through the Raf/MEK/ERK, PI3K, and Ral-GDS pathways were used, as were selective pathway inhibitors. The results show that *ODC* transcription was controlled through a pathway dependent on Raf/MEK/ERK activation, whereas activation of the PI3K, Raf/MEK/ERK and p38 MAPK pathways were necessary for translational regulation of ODC. The p38 MAPK pathway had a negative role in translational regulation, whereas the other two stimulated translation. The importance of the Raf/MEK/ERK pathway in ODC activation was also shown in a transgenic mouse line overexpressing a constitutively active mutant of MEK1 driven by the keratin 14 promoter *(45)*.

MAPK involvement in polyamine catabolism through the induction of spermine/spermidine-N^1-acetyltransferase has also been investigated *(46)*. An activated Ki-*ras* was expressed in CaCo2 cells and ODC activity was increased, as has been shown by others. The cells showed decreased expression of spermine/spermidine-N^1-acetyltransferase via a mechanism involving Raf/MEK/ERK-dependent downregulation of PPARγ.

5. Conclusion

It is clear that stimulation of polyamine biosynthesis followed by increased polyamine levels are required for efficient cell-cycle progression after mitogenic stimulation *(2,47–49)*. As reviewed previously, it is also clear that polyamines are required for normal expression of a number of transcription factors after mitogenic stimulation, one of which is c-Myc. Importantly, c-Myc is a transcription factor for *ODC (5)*. It is also evident that several signal transduction pathways are involved in induction of balanced polyamine biosynthesis. Altogether, the data indicate that polyamines are required for efficient signal transduction and that ODC induction by c-Myc provides a feedback pathway to ensure that the polyamine levels are sufficiently high for efficient signal transduction (Fig. 2). Such a positive loop involving ODC/polyamines and MAPK pathways has been suggested *(31,35)*. Thus polyamines appear to be important both in and downstream of signal transduction pathways. However, a necessary goal for polyamine research is to find the specific molecular targets for polyamine function in signal transduction as a final proof of concept.

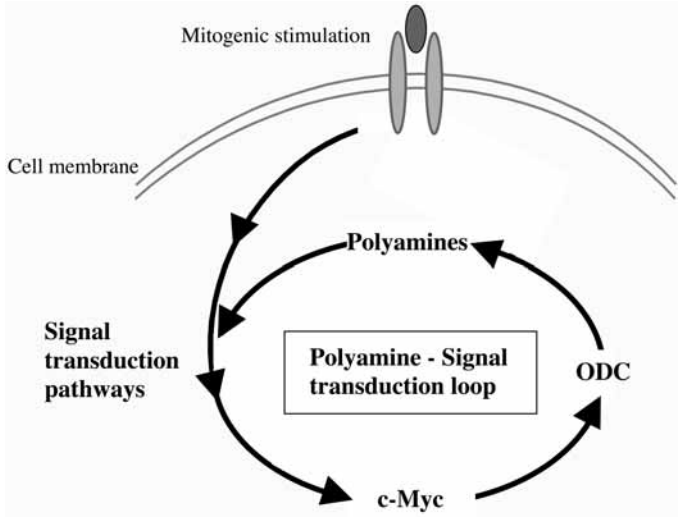

Fig. 2. Schematic representation of a polyamine signal transduction loop.

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References

1. Thomas, T. and Thomas, T. J. (2001) Polyamines in cell growth and cell death: molecular mechanisms and therapeutic applications. *Cell. Mol. Life Sci.* **58,** 244–258.
2. Oredsson, S. M. (2003) Polyamine dependence of normal cell cycle progression. *Biochem. Soc. Trans.* **31,** 366–370.
3. Campbell S. L., Khosravi-Far, R., Rossman, K. L., Clark, G. J., and Der, C. J. (1998) Increasing complexity of Ras signalling. *Oncogene* **17,** 1395–1413.
4. Gutkind, J. S. (1998) Cell growth control by G protein-coupled receptors: from signal transduction to signal integration. *Oncogene* **17,** 1331–1342.
5. Bello-Fernandez, C., Packham, G., and Cleveland, J. L. (1993) The ornithine decarboxylase gene is a transcriptional target of c-Myc. *Proc. Natl. Acad. Sci. USA* **90,** 7804–7808.
6. Coleman, M. L., Marshall, C. J., and Olson, M. F. (2004) Ras and Rho GTPases in G_1-phase cell-cycle regulation. *Nat. Rev. Mol. Cell. Biol.* **5,** 355–366.
7. Nicholson, K. M. and Anderson, N. G. (2002) The protein kinase B/Akt signalling pathway in human malignancy. *Cell Signal.* **14,** 381–395.
8. Liang, J. and Slingerland, J. M. (2003) Multiple roles of the PI3K/PKB (Akt) pathway in cell cycle progression. *Cell Cycle* **2,** 339–345.
9. Zhang, W. and Liu, H. T. (2002) MAPK signal pathway in the regulation of cell proliferation in mammalian cells. *Cell Res.* **12,** 9–18.
10. Bulavin, D. V. and Fornace, A. J. (2004) p38 MAP kinase's emerging role as a tumor suppressor. *Adv. Cancer Res.* **92,** 95–118.

11. Weston, C. R. and Davis, R. J. (2002) The JNK signal transduction pathway. *Curr. Opin. Genet. Develop.* **12,** 14–21.
12. Zohn, I. M., Campbell, S. L., Khosravi-Far, R., Rossman, K. L., and Der, C. J. (1998) Rho family proteins and Ras transformation: the RHOad less travelled gets congested. *Oncogene* **17,** 1415–1438.
13. Heim, M. H. (1999) The Jak-STAT pathway: cytokine signalling from the receptor to the nucleus. *J. Recept. Signal Transduct. Res.* **19,** 75–120.
14. Akhurst, R. J. and Derynck, R. (2001) TGF-β signalling in cancer—a double-edged sword. *Trends Cell. Biol.* **11,** S44–S51.
15. Metcalf, B. W., Bey, P., Danzin, C,. Jung, M. J., Casero, P., and Vevert, J. (1978) Catalytic irreversible inhibition of mammalian ornithine decarboxylase (E.C.4.1.1.17) by substrate and product analogs. *J. Am. Chem. Soc.* **100,** 2551–2553.
16. Celano, P., Baylin, S. B., Giardiello, F. M., Nelkin, B. D., and Casero, R. A. (1988) Effect of polyamine depletion c-myc expression in human colon carcinoma cells. *J. Biol. Chem.* **263,** 5491–5494.
17. Wang, J.-Y., McCormack, S. A., Viar, M. J., et al. (1993) Decreased expression of protooncogenes c-*fos*, c-*myc*, and c-*jun* following polyamine depletion in IEC-6 cells. *Gastrointest. Liver Physiol.* **54,** 3212–3217.
18. Patel, A. R. and Wang, J.-Y. (1997) Polyamines modulate transcription but not post-transcription of c-*myc* and c-*jun* in IEC-6 cells. *Am. J. Physiol.* **42,** C1020–C1029.
19. Celano, P., Baylin, S. B., and Casero, R. A. (1989) Polyamines differentially modulate the transcription of growth-associated genes in human colon carcinoma cells. *J. Biol. Chem.* **264,** 8922–8927.
20. Tabib, A. and Bachrach, U. (1994) Activation of the proto-oncogenes c-*myc* and c-*fos* by c-*ras*: involvement of polyamines. *Biochem. Biophys. Res. Commun.* **212,** 720–727.
21. Wang, H. and Scott, R. E. (1992) Induction of c-jun independent PKC, pertussis toxin-sensitive G protein, and polyamines in quiescent SV40-transformed 3T3 T cells. *Exp. Cell. Res.* **203,** 47–55.
22. Schulze-Lohoff, E., Fees, H., Zaner, S., Brand, K., and Sterzel, R. B. (1994) Inhibtion of immediate-early-gene induction in renal mesangial cells by depletion of intracellular polyamines. *Biochem. J.* **298,** 647–653.
23. Charollais, R. H. and Mester, J. (1988) Resumption of cell cycle in BALB7c-3T3 fibroblasts arrested by polyamine depletion: relation with “competence” gene expression. *J. Cell. Physiol.* **137,** 559–564.
24. Kramer, D. L., Fogel-Petrovic, M., Diegelman, P., et al. (1997) Effects of novel spermine analogues on cell cycle progression and apoptosis in MALME-3M human melanoma cells. *Cancer Res.* **57,** 5521–5527.
25. Kramer, D. L., Vujcic, S., Diegelman, P., et al. (1999) Polyamine analogue induction of the p53-p21WAF1/CIP1-Rb pathway and G1 arrest in human melanoma cells. *Cancer Res.* **59,** 1278–1286.
26. Bauer, P. M., Buga, G. M., and Ignarro, L. J. (2001) Role of p42/p44 mitogen-activated kinase and p21waf1/cip1 in the regulation of vascular smooth muscle cell proliferation by nitric oxide. *Proc. Natl. Acad. Sci. USA* **98,** 12,802–12,807.
27. Sistonen, L., Holtta, E., Lehvaslaiho, H., Lehtola, L., and Alitalo, K. (1989) Activation of the neu tyrosine kinase induces the fos/jun transcription factor complex, the glucose transporter and ornithine decarboxylase. *J. Cell. Biol.* **109,** 1911–1919.
28. Abrahamsen, M. S. and Morris, D. R. (1991) Regulation of expression of the ornithine decarboxylase gene by intracellular signal transduction pathways. In: *Bacteria to Cancer*

(Inouye, M., Campisi, J., Cunningham, D. D., and Riley, M. ed.) Wiley-Liss, Inc. NY, pp. 107–119.
29. Wang, H., Wan, J.-Y., Johnson, L. R., and Scott, R. E. (1991) Selective induction of c-*jun* and *jun*-B not c-*fos* or c-*myc* during mitogenesis in SV40-transformed cells at the prediffer-entiation growth arrest. *Cell. Growth Differ.* **2,** 645–652.
30. Flamigni, F., Marmiroli, S., Capanni, C., Stefanelli, C., Guarnieri, C., and Caldarera, C. M. (1997) Phosphatidylinosotol 3-kinase is required for the induction of ornithine decarboxy-lase in leukaemia cells stimulated to grow. *Biochem. Biophys. Res. Commun.* **239,** 729–733.
31. Flamigni, F., Facchini, A., Capani, C., Stwfanelli, C., Tantini, B., and Caldarera, C. M. (1999) p44/42 mitogen-activated protein kinase is involved in the expression of ornithine decarboxylase in leukaemia L1210 cells. *Biochem. J.* **341,** 363–369.
32. Flamigni, F., Facchini, A., Giordano, E., Tantini, E., Tantini, B., and Stefanelli, C. (2001) Signalling pathways leading to the induction of ornithine decarboxylase: opposite effects of p44/42 mitogen activated protein kinase (MAPK) and p38 MAPK inhibitors. *Biochem. Pharmacol.* **61,** 25–32.
33. Bachrach, U. and Tabib, A. (1998) Polyamines regulate protein kinase activities and signal transduction processes. COST 917 Fourth Workshop on Biogenetically Active Amines in Food, Trento, Italy, 3–7 June. p. 57.
34. Tabib, A. and Bachrach, U. (1999) Role of polyamines in mediating malignant transforma-tion and oncogene expression. *Int. J. Biochem. Cell. Biol.* **31,** 1289–1295.
35. Bachrach, U., Wang, Y. C., and Tabib, A. (2001) Polyamines; new clues to signal transduc-tion. *News Physiol. Sci.* **16,** 106–109.
36. Höltta, E., Sistonen, L., and Alitalo, K. (1988) The mechanisms of ornithine decarboxylase deregulation in c-Ha-*ras* oncogene-transformed NIH 3T3 cells. *J. Biol. Chem.* **263,** 4500–4507.
37. Höltta, E., Auvinen, M., and Andersson, L. C. (1993) Polyamines are essential for cell transformation by $pp60^{v\text{-}src}$: delineation of molecular events relevant for the transformed phenotype. *J. Biol. Chem.* **122,** 903–914.
38. Shayovitis, A. and Bachrach, U. (1995) Ornithine decarboxylase: an indicator for growth of NIH 3T3 fibroblasts and their c-Ha-ras transformants. *Biochim. Biophys. Acta* **1267,** 107–114.
39. Shantz, L. M. and Pegg, A. E. (1998) Ornithine decarboxylase induction in transformation by H-Ras and RhoA. *Cancer Res.* **58,** 2748–2753.
40. Shantz, L. M. (2004) Transcriptional and translational control of ornithine decarboxylase during Ras transformation. *Biochem. J.* **377,** 257–264.
41. Auvinen, M., Paasinen-Sohns, A., Hirai, H., Andersson, L. C., and Hölttä, E. (1995) Ornithine decarboxylase- and ras-induced cell transformation: reversal by protein kinase inhibitors and role of pp130CAS. *Mol. Cell. Biol.* **15,** 6513–6525.
42. Manni, A., Wechter, R., Gilmour, S., Verderame, M. F., Mauger, D., and Demers, L. M. (1997) Ornithine decarboxylase over-expression stimulates mitogen-activated protein kinase and anchorage-independent growth of human breast epithelial cells. *Int. J. Cancer* **70,** 175–182.
43. Manni, A., Wechter, R., Verderame, M. F., and Mauger, D. (1998) Cooperativity between the polyamine pathway and HER-2neu in transformation of human mammary epithelial cells in culture: role of the MAPK pathway. *Int. J. Cancer* **76,** 563–570.
44. Hurta, R. A. R. (2002) Altered ornithine decarboxylase and S-adenosylmethionine decar-boxylase expression and regulation in mouse fibroblasts transformed with oncogenes or con-stitutive active mitogen-activated protein (MAP) kinase. *Mol. Cell. Biochem.* **215,** 81–92.

45. Feith, D. J., Bol, D. K., Carboni, J. M., et al. (2005) Induction of ornithine decarboxylase activity is a necessary step for mitogen-activated protein kinase kinase-induced skin tumorigenesis. *Cancer Res.* **65,** 572–578.
46. Ignatenko, N. A., Babbar, N., Mehta, D., Casero, R. A., and Gerner, E. W. (2004) Suppression of polyamine catabolism by activated Ki-ras in human colon cancer cells. *Mol. Carcinogen* **39,** 91–102.
47. Fredlund, J. O. and Oredsson, M. S. (1996) Normal G_1/S transition and prolonged S phase within one cell cycle after seeding of cells in the presence of a polyamine biosynthesis inhibitor. *Cell Prolif.* **29,** 457–465.
48. Fredlund, J. O. and Oredsson, S. M. (1997) Ordered cell cycle phase perturbations in Chinese hamster ovary cells treated with an S-adenosylmethionine decarboxylase inhibitor. *Eur. J. Biochem.* **249,** 232–238.
49. Alm, K., Berntsson, P. S. H., Kramer, D., Porter, C. W., and Oredsson, S. M. (2000) Treatment of cells with the polyamine analog N^1,N^{11}-diethylnorspermine retards S phase progression within one cell cycle. *Eur. J. Biochem.* **267,** 4157–4164.

4

Cellular Signals Mediating Growth Arrest After Polyamine Depletion

Jian-Ying Wang

1. Introduction

The natural polyamines spermidine and spermine and their precursor putrescine are ubiquitous organic cations of low molecular weight in eukaryotic cells and are intimately involved in distinct cellular functions *(1,2)*. For many years, regulation of cellular polyamines has been recognized to be the central convergence point for multiple signaling pathways driving different cellular functions. It has been shown that increased levels of cellular polyamines, either synthesized endogenously or supplied exogenously, are essential for the stimulation of cell proliferation, and that depletion of cellular polyamines inhibits cell proliferation and causes G_1 phase growth arrest in a variety of cell types *(1–3)*. To define the exact role of polyamines in cell proliferation at the molecular level, an increasing body of evidence indicates that polyamines regulate cell proliferation by virtue of their ability to modulate expression of various growth-related genes.

Polyamines are shown to positively regulate the transcription of growth-promoting genes, such as *c-fos*, *c-jun*, and *c-myc (4,5)* and negatively affect expression of growth-inhibiting genes including *p53 (6,7)*, *junD (8,9)*, and *TGFβ/TGFβ* receptor *(10,11)* through modulations at the posttranscriptional level. Increased polyamines not only stimulate expression of *c-fos*, *c-jun*, and *c-myc* genes by enhancing their messenger RNA (mRNA) synthesis, but also inhibit expression of *p53*, *junD*, and *TGFβ/TGFβ* receptor genes by increasing their mRNA degradation, resulting in the stimulation of cell proliferation. In contrast, decreased levels of polyamines lead to a reduction of the levels of growth-promoting proteins through inhibition of their gene transcription, and to increased levels of growth-inhibiting factors through stabilization of their mRNAs and proteins, which are associated with an increase in G_1 phase growth arrest. In the other words, growth inhibition after polyamine depletion is an active process that results primarily from the activation of specific cellular signals rather than a simple decrease in growth-promoting gene expression. In this chapter, we will present overview changes in cellular signals that are activated after polyamine depletion, and then analyze in some detail the mechanisms by which polyamine depletion induces the activity of these signaling pathways.

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2. Polyamines and Growth-Promoting Gene Expression

Over the past decade, four scientific developments have provided the opportunity to more exactly determine the polyamine requirement in cell cycle progression and, at the same time, to gain insight into their functions: (1) the availability of polyamine analogs with close structural and functional similarities to the natural polyamines; (2) the discovery of well-characterized inhibitors that are highly specific for key enzymes of polyamine biosynthesis; (3) the significant achievement of understanding complex regulatory pathways that control the cell cycle; and, more recently, (4) the availability of various transgenic or knockout animal models in which specific key enzymes for polyamine synthesis are either deleted or forced to overexpress in specific tissues. So far, these advances have been extended to help elucidate the polyamine requirement in cell growth and their effects on expression of cell cycle regulatory factors, including growth-promoting and growth-inhibiting genes.

A series of observations from our previous studies *(5,12,13)* and others *(4)* demonstrates that polyamines are required for the stimulation of cell proliferation at least partly through their ability to regulate proto-oncogene expression. In a gastric mucosal stress ulcer model, the repair process is associated with significant increases in the expression of proto-oncogenes *c-fos* and *c-myc* after increased polyamine synthesis *(14)*. This change in the expression of *c-fos* and *c-myc* precedes the induction of DNA synthesis after stress. Inhibition of polyamine synthesis by a-difluoromethylornithine (DFMO) decreases both proto-oncogene expression and cell division. In cultured normal intestinal epithelial cells (IECs) (IEC-6 line), depletion of cellular polyamines by exposure to DFMO completely prevents the expression of *c-fos*, *c-myc*, *c-jun*, and polyamine replacement by exogenously adding spermidine, which reverses the inhibitory effects of DFMO on the expression of proto-oncogenes *(5)*. These data indicate that increased expression of proto-oncogenes is involved in early modulation of mucosal growth and may be part of the mechanism responsible for polyamine-stimulated cell division. In support of this possibility, we *(12,13)* and others *(4)* have further demonstrated that polyamines are necessary for stimulation of transcription of the proto-oncogenes *c-myc*, *c-fos*, and *c-jun* and that polyamine depletion-mediated decreases in mRNAs levels of these genes are primarily caused by decreased levels of gene transcription.

Cells, however, still maintain a high basal level of proto-oncogene expression after inhibition of polyamine synthesis in different types of cells *(4,5)*. Also, the rate of cell proliferation in polyamine-deficient cells is dramatically lower than that of control cells *(5,14,15)*. These results suggest that growth inhibition after polyamine depletion must result from a mechanism other than a simple decrease in proto-oncogene expression.

3. Polyamine Depletion Activates p53 Signaling Pathway

3.1. Polyamine Depletion Associated With Increased p53 *Gene Expression*

Considerable interest over the past several years has been devoted to the mechanisms of negative growth control. In the small intestinal mucosa, for example, mitotic activity is confined to the crypt region and must be finely tuned to the rapid rate of loss of mature enterocytes from the villous tip. Theoretically, mucosal epithelial cell

turnover is controlled by both positive and negative control mechanisms, with stimulation or suppression of cell renewal mediated through growth-promoting or growth-inhibiting genes. Although growth-promoting genes have been extensively studied in vivo and in vitro, studies to elucidate the role and significance of negative growth control in the regulation of cell growth have recently started.

The *p53* gene encodes for a nuclear phosphoprotein, which was originally discovered as a cellular protein bound to the SV40 T antigen in transformed cells. p53 is present in low concentrations in normal cells and has a half-life of 6–20 min *(16,17)*. Expression of the *p53* gene is highly regulated by the cell according to its state of growth. Steady-state levels of p53 mRNA are significantly increased in certain growth conditions and in cells transformed by a variety of means, including viral infection, chemical treatment, and transfection of oncogenes *(18,19)*. Inactivation of the *p53* gene occurs in more than half of all human tumors, implying that expression of the *p53* gene has an important physiological role in the control of the cell cycle and that loss of this gene represents a fundamental step in the pathogenesis of cancer *(16,17)*. This action of p53 has been ascribed to its ability to induce expression of a cellular gene WAF1/CIP1 that encodes a 21-kDa inhibitor of G_1 cyclin-dependent kinases *(17,19)*. In addition, there is increasing evidence that the p53 protein also induces apoptosis *(16)*. Activation of the *p53* gene expression induces growth arrest, apoptosis, or both.

The observation that p53 protein can suppress cell growth suggests that p53 may play an important role in the control of the cell cycle, which regulates the orderly flow of the cell in and out of the G_1 phase during normal cell proliferation *(16–20)*. p53 functions as a transcriptional factor and is a cell-cycle control protein because progression from the G_1 to S phase is often blocked in cells expressing high p53 protein levels *(17)*. Mercer and colleagues *(21)* demonstrated that induction of p53 expression by transfecting a conditional p53 expression plasmid inhibits cell-cycle progression and that, when growth-arrested cells are stimulated to proliferate, induction of p53 expression inhibits G_0/G_1 progression into the S phase. p53 also is required for the G_1 arrest caused by g-ray exposure *(16,17)*.

The first evidence showing the role of the *p53* gene expression in growth inhibition after polyamine depletion is from our observations *(6,20)* and others *(22,23)* in normal IECs. Using cultured IEC-6 cells, we have made the unique observation that inhibition of polyamine synthesis with DFMO significantly increases *p53* gene expression, which is associated with an increase in G_1 phase growth arrest but not apoptosis *(6)*. Increases in p53 after polyamine depletion were associated with increases in other cell-cycle inhibitors, including $p21^{Waf1/Cip1}$ and $p27^{Kip1}$. In control cells, steady-state levels of p53 mRNA were present at 4 d and then almost completely disappeared at 6 d, with minimal expression at 12 d after plating. Depletion of cellular polyamine through DFMO treatment significantly increased expression of the *p53* gene (Fig. 1). This increase in mRNA levels for the *p53* gene was noted at 4 d and remained elevated at 12 d after exposure to DFMO. Maximum increases in p53 mRNA levels occurred between 6 and 12 d after addition of DFMO and were more than 10 times that of control values. The increased levels of p53 mRNA in polyamine-deficient cells were paralleled by increases in p53 protein. Spermidine given together with DFMO completely prevented the increased

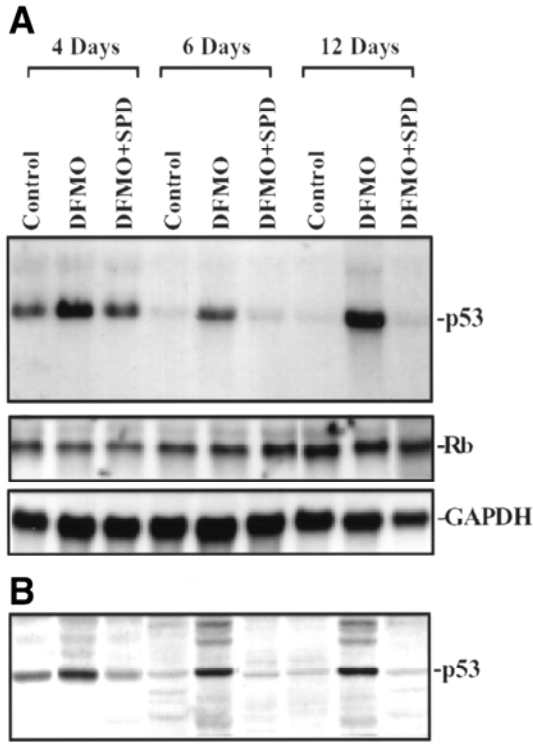

Fig. 1. Expression of p53 in control IEC-6 cells and cells exposed to 5 m*M* a-difluoromethylornithine (DFMO) or DFMO plus spermidine (SPD). **(A)** Representative autoradiograms from control cells and cells exposed to DFMO or DFMO + SPD for 4, 6 and 12 d. Total cellular RNA was isolated and mRNA levels were examined by Northern blot analysis using *p53* and *Rb* gene complimentary DNA probes. **(B)** Representative immunoblots for p53 protein as measured by Western blot analysis. p53 protein was identified by probing nitrocellulose with a specific anti-p53 antibody. Three experiments were performed that showed similar results.

expression of the *p53* gene. The concentrations of p53 mRNA and protein in cells treated with DFMO plus spermidine were indistinguishable from those in cells grown in control cultures. The effect of polyamines on *p53* gene expression is specific because polyamine depletion did not induce expression of the *Rb* gene in IEC-6 cells. These results suggest that p53 is involved in the regulation of intestinal mucosal growth by polyamines.

Consistent with our findings, Kramer and colleagues *(17,22)* have reported that exposure of human melanoma cells (MALME-3M cell) to a polyamine analog, N^1,N^{11}-diethylnorspermdine (DENSPM), not only decreases cellular polyamines but also increases *p53* and *p21* gene expression. DENSPM is known to deplete polyamine pools by both inhibiting biosynthetic enzymes and potently inducing the polyamine catabolic enzyme spermidine/spermine N^1-acetyltransferase. Treatment with DENSPM increases

wild-type p53 (approx 10-fold at maximum), which is concomitant with an increase in p21 in MALME-3M cells. Another cyclin-dependent kinase inhibitor, p27, and cyclin D1 increase slightly, whereas proliferating cell nuclear antigen and p130 remain unchanged. Induction of p21 protein is paralleled by an increase in its mRNA but induction of p53 protein is not, suggesting that cellular polyamines dictate transcriptional activation of the *p21* gene and posttranscriptional regulation of the *p53* gene. Inconsistent with observations in IEC-6 cells, polyamine depletion by DENSPM in MALME cells also causes an increase in hypophosphorylated Rb protein.

In addition, polyamines also regulate extracellular signal-regulated kinase (ERK) activity in IEC-6 cells *(23)*. Polyamine depletion results in an approx 50% increase in ERK-1 activity, and supplementation with putrescine restores the basal level of ERK-1 activity. Polyamine depletion has only a marginal effect on the level of ERK-1 protein. On the other hand, polyamine depletion increases ERK-2 activity 150% compared with control in IEC-6 cells. This increase in ERK-2 is largely prevented by addition of putrescine to DFMO-treated cells. Interestingly, polyamine depletion significantly reduced the level of ERK-2 protein. Similarly, the activity of the c-Jun NH_2-terminal kinase is elevated on polyamine depletion and remains elevated in the presence of putrescine. The level of JNK protein is low in polyamine-depleted cells and returns to normal with the addition of putrescine. Inhibition of polyamine synthesis also induces STAT3 tyrosine phosphorylation in IEC-6 cells *(24)*. However, the exact roles of polyamine depletion-modulated ERK and c-Jun NH_2-terminal kinase activities and STAT3 phosphorylation in regulation of p53 and p21 expression remain to be elucidated.

3.2. Polyamine Depletion Stabilizes p53 mRNA

In response to polyamine depletion, cellular levels of p53 protein are greatly increased, and the ability of p53 to bind specific DNA sequences is significantly activated *(16,17,25,26)*. Because expression of the *p53* gene is rapid and transient, it acts as a mediator, linking short-term stress signals to growth arrest or apoptosis by regulating the activation of specific genes. p53 is an ephemeral protein, and its half-life is approx 25–40 min in variety of cell types *(17)*. In addition to transcriptional regulation, expression of the *p53* gene is primarily regulated at the posttranscriptional level. Increasing evidence suggests that phosphorylation plays a major role in the regulation of both the stability of p53 protein and its DNA-binding activity. It has been shown that phosphorylation of human p53 at Ser-15 and Ser-20 induces conformational changes in the NH_2 terminus that disrupt Mdm2 binding and lead to its stabilization *(16,17)*.

We have recently investigated the mechanisms of *p53* gene expression by cellular polyamines in normal IECs *(6,20)*. Polyamine depletion by treatment with DFMO dramatically increases the stability of p53 mRNA as measured by the mRNA half-life, but has no effect on *p53* gene transcription in IEC-6 cells. As can be seen in Fig. 2, p53 mRNA levels in control cells declined rapidly after inhibition of gene transcription by addition of actinomycin D. The half-life of p53 mRNA in control cells was approx 45 min. However, the stability of p53 mRNA was dramatically increased by polyamine depletion with a half-life of longer than 18 h. Increased half-life of p53

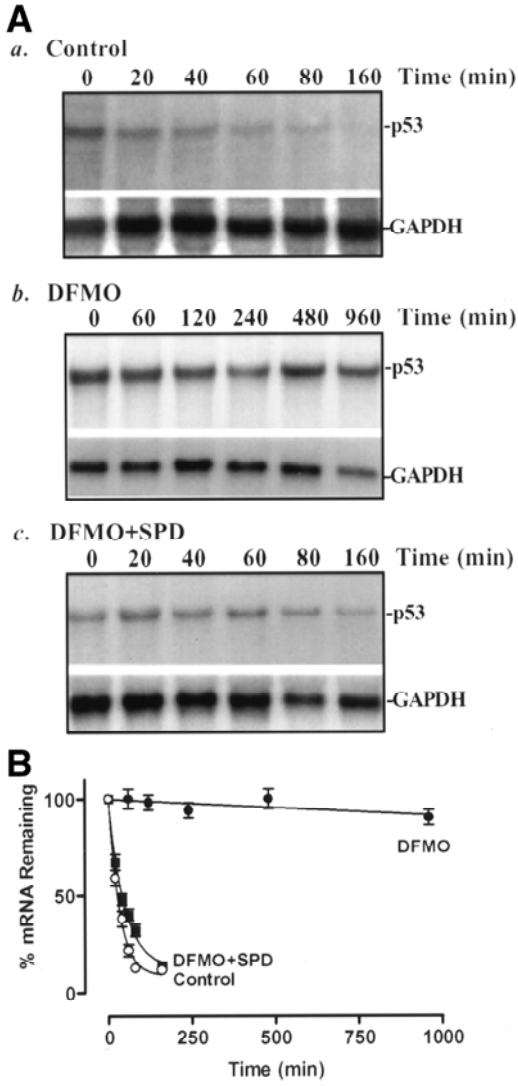

Fig. 2. Cytoplasmic half-life studies of p53 mRNA from IEC-6 cells in the presence or absence of cellular polyamines. **(A)** Representative autoradiograms of Northern blots. Cells were untreated (*a*) or treated with DFMO alone (*b*) or DFMO plus SPD (*c*) for 6 d and then incubated with 5 m*M* actinomycin D/mL for indicated times. Total cellular RNA was isolated and p53 mRNA levels were assayed by Northern blot analysis using a p53 complimentary DNA probe. Loading of RNA was monitored by hybridization to labeled GAPDH probe. **(B)** Percent of p53 mRNA remaining in IEC-6 cells described in **A**. Values are means ± SE of data from three separate experiments, and relative levels of p53 mRNA were corrected for RNA loading as measured by densitometry of GAPDH.

mRNA was prevented when spermidine was given together with DFMO. The half-life of p53 mRNA in cells treated with DFMO plus spermidine was approx 48 min, similar to that of controls (without DFMO). In contrast, inhibition of polyamine synthesis did not increase *p53* gene transcription as measured by using nuclear run-on transcription assays *(20)*. There were no significant differences in the rate of *p53* gene transcription between control cells and cells exposed to DFMO in the presence or absence of spermidine for 6 d. These findings clearly indicate that polyamines regulate the *p53* gene expression posttranscriptionally in IECs, and that depletion of cellular polyamines induces p53 mRNA levels primarily through the increase in its stability.

Posttranscriptional regulation, especially modulation of mRNA stability, has been shown to play an important role in gene expression *(18,19)*. The turnover rate of a given mRNA can be determined by interactions of *trans*-acting factors with specific *cis*-element(s) located within 3¢-untranslated regions (3¢ UTR) *(27–29)*. Many labile mRNAs including those that encode transcription factors contain AU-rich elements in their 3¢ UTR. The presence of a reiterated pentamer (5¢-AUUUA-3¢) in many AU-rich elements is associated with rapid mRNA turnover and translation attenuation *(27)*. Deletion of the AU-rich element region enhances mRNA stability and insertion of the region into the 3¢ UTR of a normally stable globin mRNA significantly destabilizes it *(27–29)*. Several AUUUA-binding proteins have been identified in different cell types, although the mechanisms of how these proteins affect mRNA turnover are unclear. It is unclear at present that an AU-rich element region in the p53 3¢ UTR is critical for the stabilization of p53 mRNA after polyamine depletion in IECs.

3.3. Polyamine Depletion Stabilizes p53 Protein Through the Interaction With Nucleophosmin

Our previous studies show that polyamine depletion also stabilizes p53 protein in normal IECs *(20)*. The levels of p53 protein in control cells declined rapidly after inhibition of protein synthesis by administration of cycloheximide. The half-life of p53 protein in control IEC-6 cells was approx 15 min and increased to approx 38 min in cells exposed to DFMO for 6 d. When DFMO was given together with spermidine, p53 protein levels decreased at the rate similar to that observed in controls, with a half-life of approx 18 min. These data indicate that cellular polyamines are essential for the degradation of p53 protein in normal IECs and that induced accumulation of p53 protein in polyamine-deficient cells results, at least partially, from the protein stabilization.

Nucleophosmin (NPM) is a multifunctional protein that was originally identified as a nucleolar protein involved in ribosome biogenesis and has been recently shown to regulate p53 activity *(30,31)*. We have demonstrated that polyamines modulate NPM activity in IEC-6 cells *(32)*. Depletion of cellular polyamines by DFMO stimulates expression of the *NPM* gene and induces nuclear translocation of NPM protein. Polyamine depletion stimulates NPM expression primarily by increasing *NPM* gene transcription and its mRNA stability, and induced NPM nuclear translocation through activation of phosphorylation of mitogen-activated protein kinase. Increased NPM physically interacts with p53 and forms a NPM/p53 complex in polyamine-deficient

cells. Inhibition of NPM expression by small interfering RNA (siRNA) targeting of a specific site on the NPM mRNA coding region not only destabilizes p53, as indicated by a decrease in its protein half-life, but also prevents the increased p53-dependent transactivation as indicated by a decrease in p21-promoter activity.

As shown in Fig. 3, transfection with NPM siRNA prevented increased NPM in polyamine-deficient IEC-6 cells. Levels of NPM protein were decreased by approx 75% when polyamine-deficient cells were exposed to NPM siRNA for 48 h. Transfection with control siRNA (C-siRNA) at the same concentrations for 48 h showed no inhibitory effects. Inhibition of NPM expression by NPM siRNA also prevented the increased levels of nuclear p53 protein in polyamine-deficient cells. Nuclear p53 protein was decreased by approx 70% after inhibition of NPM by treatment with NPM siRNA for 48 h. Consistently, decreased p53 by NPM siRNA was associated with an inhibition of p53-dependent transcriptional activity as indicated by a decrease in p21 promoter activity. The level of p21-promoter activity in DFMO-treated cells transfected with NPM siRNA was identical with that obtained from controls. Treatment with C-siRNA had no effect on levels of nuclear p53 protein and p53 transcriptional activity in polyamine-deficient cells. These findings strongly suggest that induced NPM after polyamine depletion stabilizes p53 protein and regulates p53 transcriptional activity in normal IECs.

3.4. Increased p53 Causes Growth Inhibition in Polyamine-Deficient Cells

The following three studies provide evidence showing that increased accumulation of p53 protein plays a critical role in the process of growth inhibition after polyamine depletion in normal IECs. In the first study, the time course of *p53* gene expression and cell growth were examined after polyamine-deficient cells were exposed to exogenous spermidine *(6,20)*. IEC-6 cells were initially treated with DFMO for 4 d and then exposed to spermidine. The changes in both *p53* gene expression and cellular DNA synthesis were measured at various times after spermidine was added to the medium containing DFMO. Depletion of cellular polyamines by administration of DFMO for 4 d increased the steady-state levels of p53 mRNA and protein. When these polyamine-deficient cells were exposed to exogenous spermidine, prevention of increased p53 mRNA and protein preceded the beginning of cellular DNA synthesis as measured by [^{3}H]-thymidine incorporation. The significant decreases in p53 mRNA and protein levels were observed 12 h after exposure to exogenous spermidine, whereas the increased rate of DNA synthesis occurred at 24 h after administration of spermidine. These findings indicate that the restoration of cell growth by exogenous spermidine in polyamine-deficient cells is preceded by a decrease in *p53* gene expression.

In the second study, the effect of inhibition of p53 expression by using p53 antisense oligodeoxyribonucleotides on cell growth was examined in polyamine-deficient IEC-6 cells *(20)*. Cells were grown in the presence of DFMO for 4 d and DNA synthesis was assayed by [^{3}H]-thymidine incorporation 48 h after administration of different concentrations of p53 antisense or sense oligomers. Exposure of polyamine-deficient cells to p53 antisense oligomers significantly increased DNA synthesis in the presence of DFMO. Treatment with p53 antisense oligomers also inhibited p53 protein expres-

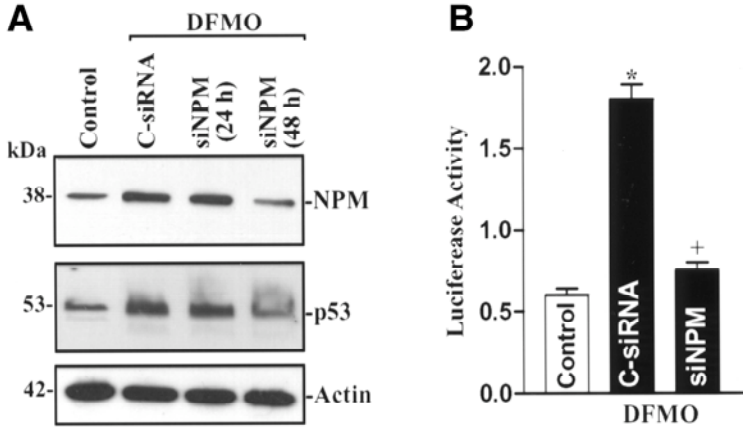

Fig. 3. Effects of inhibition of nucleophosmin (NPM) expression by treatment with the siRNA-targeting NPM mRNA-coding region (siNPM) on levels of p53 protein and its transcriptional activity in polyamine-deficient cells. **(A)** Representative immunoblots for NPM and p53 proteins. Cells were grown in the culture containing DFMO for 4 d and transfected with either control siRNA (C-siRNA) or siNPM by lipofectamine technique. Cells were harvested 48 h after transfection in the presence of DFMO, and levels of NPM and p53 proteins were measured by Western blot analysis. Equal loading of proteins was monitored by actin immunoblotting. **(B)** Changes in p53 transcriptional activity as measured by p21 promoter activity in cells described in **(A)**. Cells were exposed to DFMO for 4 d and then transfected with p21-promoter luciferase reporter plasmid and either siNPM or C-siRNA. After 48 h of incubation in the presence of DFMO, transfected cells were harvested and assayed for luciferase activity. Data of p21 promoter activity were normalized by b-galactosidase activity from cotransfection plasmid pRSV b-galactosidase. Values are means ± SE of data from six dishes. *, + $p < 0.05$ compared with controls and DFMO-treated cells transfected with C-siRNA, respectively.

sion in DFMO-treated cells. When DFMO-treated cells were exposed to 2 m*M* antisense oligomers for 48 h, increased p53 protein levels were prevented as measured by Western blot analysis and immunohistochemical staining. Treatment with sense p53 oligomers at the same concentrations, however, showed no significant effects on DNA synthesis and p53 protein expression. These results provide direct evidence that increased expression of the *p53* gene plays a critical role in growth inhibition of normal intestinal epithelial cells after polyamine depletion.

In the third study, the influence of decreased p53 stability and transcriptional activity by inhibiting NPM expression on cell proliferation was examined in polyamine-deficient IEC-6 cells. Increased NPM in DFMO-treated cells was associated with a significant decrease in cell growth, which was completely prevented by exogenous putrescine *(32)*. In studies using NPM siRNA, cells were initially grown in the medium containing DFMO for 4 d, and then transfected with NPM siRNA or C-siRNA. Cell numbers were assayed 48 h after transfection in the presence of DFMO and minimal putrescine (0.5 m*M*). Decreased levels of p53 through inhibition of NPM by transfec-

tion with NPM siRNA significantly promoted cell proliferation in polyamine-deficient cells. The results suggest, for the first time, that NPM/p53 signaling is involved in negative control of normal IEC growth after polyamine depletion.

3.5. A Novel Model Delineating the Negative Control of Gut Mucosal Growth After Polyamine Depletion

Based on our previous studies *(6,15,20,32–34)* and others *(2,23,24,35,36)*, we propose a model delineating the regulation of expression of the *p53* gene by cellular polyamines and the involvement of p53 in the process of growth inhibition of normal small intestinal mucosa after polyamine depletion (Fig. 4). In this model, polyamines negatively regulate posttranscriptional regulation of the *p53* gene, and the activation of p53 expression plays a critical role in the negative control of the small intestinal mucosal growth. Depletion of cellular polyamines, either by inhibition of their synthesis, stimulation of catabolism, or suppression of polyamine uptake, enhances expression of the *p53* gene via both the stabilization of p53 mRNA and the decrease in p53 protein degradation. Although the exact mechanism by which polyamine depletion stabilizes p53 mRNA remains unclear, our previous studies have demonstrated that induced NPM interacts with and stabilizes p53 protein in polyamine-deficient cells. The resultant accumulation of p53 after polyamine depletion activates the transcription of cell-cycle arrest genes such as *p21*, which then blocks the G_1–S phase transition, decreases proliferation, and inhibits mucosal growth of the small intestine.

4. Activation of JunD/AP-1 Activity After Polyamine Depletion

4.1. Polyamine Depletion Associated With Increase in JunD/AP-1 Activity

JunD is a member of the *jun* family proto-oncogenes, which are primary components of the activator protein-1 (AP-1) transcription factor *(37,38)*. The Jun proteins (c-Jun, JunB, and JunD) are basic leucine transcription factors that can form either AP-1 homodimers (Jun/Jun) or AP-1 heterodimers with members of related the Fos family (c-Fos, FosB, Fra-1, and Fra-2) or the ATF family (ATF2, ATF3, and ATF4) *(38)*. Jun/Jun and Jun/Fos dimers bind to the TPA responsive element TGACTCA present in many gene promoters, whereas Jun/ATF dimers bind preferentially to the cyclic AMP responsive element TGACGTCA *(39)*. The three Jun proteins are similar in their DNA-binding affinity that is determined by the C-terminal leucine zipper dimerization domain *(40)*. The N-terminal transactivation domain is less conserved and may account for the different transactivation characteristics of the Jun proteins. There is increasing evidence that individual AP-1 dimers play distinct functions in different cellular contexts, including cell proliferation, growth arrest, differentiation, and apoptosis *(37–40)*. For example, *c-jun* and *junB* function as immediate early response genes and activation of these two genes enhances the transition from a quiescent state to a proliferating state, indicating that c-Jun and JunB are positive AP-1 factors for cell proliferation. In contrast, the activation of *junD* gene expression slows cell proliferation in some cell types and increases the percentage of population of cells arrested in the G_0/G_1 phase of

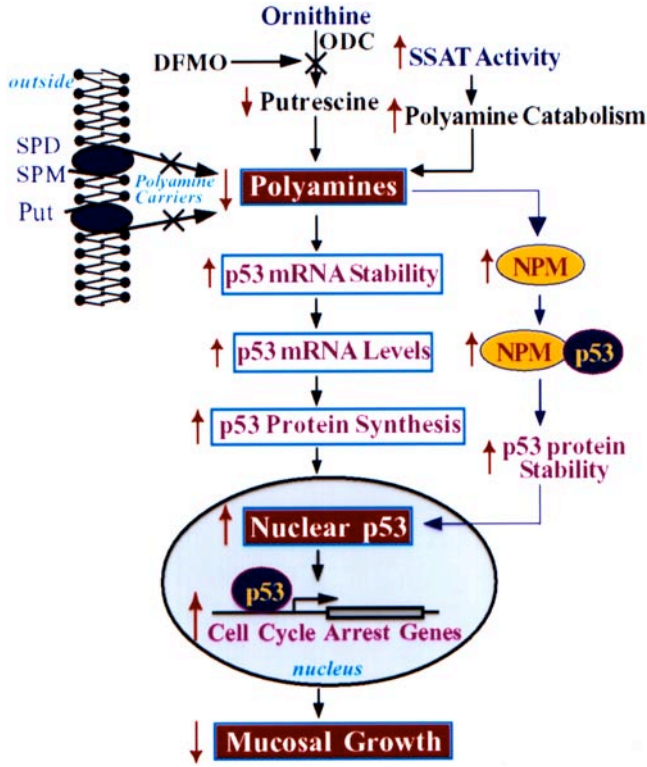

Fig. 4. Schematic diagram depicting the regulation in expression of the *p53* gene by cellular polyamines and the proposed role of p53 in growth inhibition of the small intestinal mucosa after polyamine depletion. Cellular polyamine levels are decreased by either inhibition of their biosynthesis by blocking ornithine decarboxylase activity, stimulation of the catabolism through the activation of spermidine/spermine-*N*-acetyltransferase activity, or suppression of polyamine uptake. Decreased cellular polyamines stabilize p53 messenger RNA and result in the accumulation of p53 through the induction of newly synthesized p53 protein. Polyamine depletion also increases nucleophosmin that interacts with and stabilizes p53 protein. The resultant increases in p53 initiate or enhance transcription of cell-cycle arrest genes such as *p21*, inhibits cell proliferation, and suppresses mucosal growth of the small intestine. SPD, spermidine; SPM, spermine; Put, putrescine; DFMO, a-difluoromethylornithine; ODC, ornithine decarboxylase; SSTA, spermidine/spermine-*N*-acetyltransferase; NPM, nucleophosmin.

the cell cycle *(37,41)*, suggesting that JunD is a negative AP-1 factor and downregulates the G_1 to S phase transition.

Patel and Wang *(9)* have demonstrated that depletion of cellular polyamines is associated with an increase in JunD/AP-1 activity in IECs. It has been shown that exposure of IEC-6 cells or Caco-2 (a human colon carcinoma cell line) to DFMO for 4 and 6 d significantly increases AP-1-binding activity as measured by electrophoretic mobility shift assays. Spermidine, when given with DFMO, restores AP-1-binding activity toward

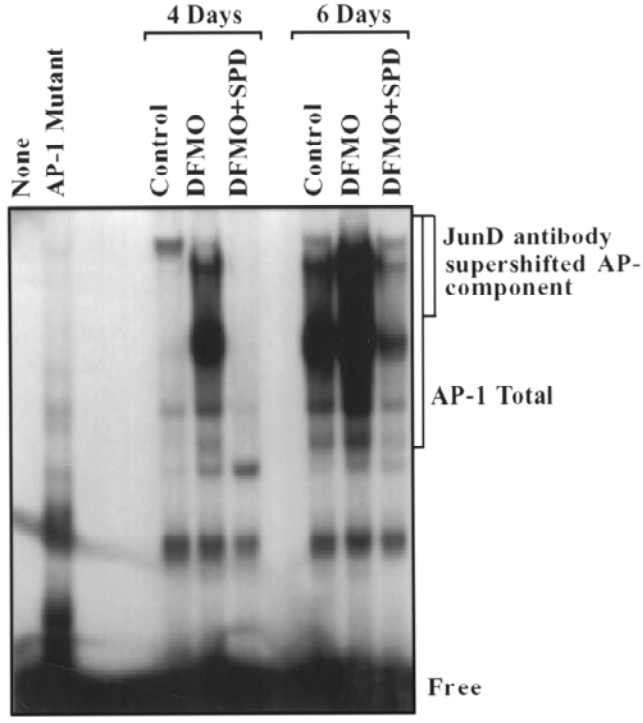

Fig. 5. Effect of addition of the antibody against JunD protein on AP-1 composition in cells exposed to DFMO alone or DFMO plus SPD. Gel supershift assays were performed by initially incubating nuclear protein with 32p end-labeled oligonucleotides containing a single AP-1-binding site. The antibody against JunD protein was then added to the binding reaction mixture. The positions of supershifted AP-1 components, the total AP-1-binding activity, and freely migrating probes are indicated. No antibody: the lane with nuclear extract and no antibody. Control antibody: the reaction mixture with nuclear extracts and the control antibody (anti-Myc antibody). Three experiments were performed that showed consistent results.

normal. As can be seen in Fig. 5, the anti-JunD antibody, when added to the binding reaction mixture, dramatically supershifted the AP-1 complexes present in the IEC-6 cell exposed to DFMO for 4 and 6 d. The AP-1 activity attributed to JunD in the DFMO-treated cells was approximately one-third of the total AP-1-binding activity on d 4, and about half on d 6, respectively. In control cells and cells exposed to DFMO and spermidine, the AP-1-binding activity was slightly supershifted by the anti-JunD antibody. On the other hand, addition of antibodies against c-Jun or JunB to the binding reaction mixture had an effect on the AP-1-binding activity in all three treatment groups. There was no stimulation of the AP-1-activity attributed to c-Jun or JunB by DFMO (*5,9*). Although the anti-Fos antibody also partially supershifted the AP-1 complexes, there were no significant differences in the AP-1 activity attributed to Fos between control cells and cells exposed to DFMO or DFMO plus spermidine. The increased AP-1-

binding activities in polyamine-deficient cells were not supershifted by the anti-Myc antibody. These results indicate that the increase in AP-1 activity in polyamine-deficient cells is primarily contributed by an increase in JunD/AP-1, whereas c-Jun/AP-1 and JunB/AP-1 activity remains essentially unchanged or decreased.

4.2. Polyamine Depletion Fails to Induce junD *Gene Transcription but Stabilizes JunD mRNA*

The increased JunD/AP-1 activity after polyamine depletion is primarily the result of activation of *junD* gene expression in IECs *(8)*. There are significant increases in JunD mRNA and protein in polyamine-deficient IEC-6 cells *(9)*, although expression of the *c-fos*, *c-jun*, and *junB* genes are decreased *(5,12)*. Increased JunD mRNA levels were approx 6.6 times the control levels on d 4 and approximately nine times on d 6 after treatment with DFMO. Increased levels of JunD mRNA were paralleled by a significant increase in JunD protein, which was clearly located in the nucleus. These increases in both JunD mRNA and protein in DFMO-treated cells were completely prevented by addition of exogenous spermidine.

To test the possibility that the increase in JunD mRNA level in polyamine-deficient cells results from an increase in the mRNA synthesis, we have examined changes in the rate of *junD* gene transcription by using nuclear run-on transcription assay *(8)*. Our results showed that inhibition of polyamine synthesis by DFMO did not increase *junD* gene transcription in IEC-6 cells. There were no significant differences in the rate of *junD* gene transcription between control cells and cells exposed to DFMO in the presence or absence of spermidine for 4 and 6 d. We also examined the rapid effect of addition of exogenous spermidine on *junD* gene transcription in control cells and demonstrated that exposure of normal IEC-6 cells (without DFMO) to 5 m*M* spermidine for 2 and 4 h did not alter the rate of *junD* gene transcription. On the other hand, rates of *c-myc* and *c-jun* gene transcription were significantly decreased after polyamine depletion *(12)*. These results clearly indicate that cellular polyamines play only a minor role in regulation of the *junD* gene transcription and that the increase in steady-state levels of JunD mRNA after polyamine depletion is related to a mechanism other than the stimulation of *junD* gene transcription in normal IECs.

To determine the involvement of posttranscriptional regulation in this process, JunD mRNA stability was examined by measurement of the mRNA half-life. Depletion of cellular polyamines by DFMO increased the stability of JunD mRNA in IEC-6 cells *(8)*. In control cells, JunD mRNA levels declined rapidly after inhibition of gene transcription by addition of actinomycin D. The half-life of JunD mRNA in control cells was approx 50 min. However, the stability of JunD mRNA was significantly increased by polyamine depletion with a half-life of more than 4 h. The natural polyamine spermidine, hen given together with DFMO, almost completely prevented the increased half-life of JunD mRNA in polyamine-deficient cells. The half-life of JunD mRNA in cells exposed to DFMO plus spermidine was approx 60 min, similar to that of controls (without DFMO). These findings indicate that polyamines regulate the *junD* expression posttranscriptionally in IECs and that depletion of cellular polyamines induces JunD mRNA levels primarily by increasing its stability.

4.3. Increased JunD/AP-1 Inhibits Cell Proliferation Through p21

The following three logically linked studies show that increased JunD/AP-1 inhibits IEC proliferation at least partially through the activation of the p21 promoter after polyamine depletion *(8,32)*. The first study examined the association of increased JunD/AP-1 activity with *p21* gene expression after polyamine depletion. Consistent on its stimulatory effect on JunD/AP-1 activity, depletion of cellular polyamines by DFMO also activated the p21 promoter, induced its mRNA expression, and increased levels of p21 protein in IEC-6 cells. The activity of p21 promoter luciferase reporter was greater than four times the control values on d 6, and increased p21 mRNA levels in the DFMO-treated cells were approximately six times the control levels. In the presence of DFMO, addition of exogenous spermidine not only blocked the induction of JunD/AP-1 activity, but also completely prevented increases in *p21* gene expression. These results indicate that induced JunD/AP-1 activity after polyamine depletion is associated with a significant increase in *p21* gene expression.

The second study tested the possibility that increased expression of *p21* gene expression is because of the activation of JunD/AP-1 activity after polyamine depletion. The effects of blocking JunD expression by JunD antisense oligomers on p21 mRNA and promoter activity were examined in polyamine-deficient cells. Cells were grown in the presence of DFMO for 4 d and then transfected using the human p21 promoter luciferase reporter construct. JunD antisense or sense oligomers were given immediately after the transfection. p21 promoter activity was measured 48 h after incubation with JunD antisense or sense oligomers. It has been shown that decreasing JunD/AP-1 activity by treatment with JunD antisense oligomers significantly decreased p21 expression. When the polyamine-deficient cells were exposed to JunD antisense oligomers for 48 h, the p21 promoter activity was decreased, which was associated with a decrease in p21 protein. These findings suggest p21 to be a downstream target of JunD and that increased JunD/AP-1 activity after polyamine depletion inhibits IEC proliferation through a process involving p21.

The third study determined the effect of overexpression of the *junD* gene on p21 promoter activity and cell proliferation in Caco-2 cells. When cells were transfected with the expression vector containing the human *junD* complimentary DNA under the control of human cytomegalovirus immediate-early promoter (PMCV), levels of JunD protein were increased by approximately sixfold at 24 h and by approx 14-fold at 48 h after the transient transfection, respectively. The vector that lacked exogenous *junD* complimentary DNA (null) was used as a negative control and did not induce JunD levels. Overexpression of the *junD* gene significantly activated the p21 promoter. The activity of p21 promoter luciferase reporter was increased by approximately eightfold 48 h after the transfection. The increased JunD by the transfection also inhibited Caco-2 cell proliferation. Cell numbers were decreased by approx 25% at 48 h and approx 38% at 72 h after the transfection. These results indicate that increased JunD expression activates p21 promoter and plays a critical role in the negative regulation of gut mucosal epithelial cell growth.

Taken together, these studies indicate that increased JunD/AP-1 activity after polyamine depletion is implicated in the negative regulation of IEC proliferation.

Depletion of cellular polyamines stabilizes JunD mRNA, induces JunD expression, and causes the accumulation of nuclear JunD protein. Increased JunD protein leads to formation of JunD–JunD homodimers and stimulates expression of the *p21* gene through the activation of p21 promoter. The resultant increase in p21 protein suppresses activity of cyclin dependent kinases, resulting in inhibition of normal IEC proliferation in polyamine-deficient cells. In contrast, increased polyamines destabilize JunD mRNA and decrease levels of JunD protein, thus resulting in the stimulation of cell proliferation. These findings suggest that JunD/AP-1 is a natural inhibitor for normal intestinal mucosal growth and plays a critical role in negative control of epithelial cell renewal under physiological and pathological conditions. Polyamines are required for stimulation of IEC proliferation in association with their ability to enhance JunD mRNA degradation.

5. Activation of Transforming Growth Factor-β/Smad Signaling Pathway After Polyamine Depletion

5.1. Expression of the Transforming Growth Factor-β *Gene in Polyamine-Deficient Cells*

The transforming growth factor-β (TGF-β) family is a group of multifunctional peptides involved in regulation of epithelial cell growth and phenotype *(42,43)*. There are three distinct but highly related mammalian isoforms of TGFβs, named β1, -β2, and -β3. TGF-βs exert their multiple actions through heteromeric complexes of two types (type I and type II) of transmembrane receptors with a serine/threonine kinase domain in their cytoplasmic region *(44–46)*. The receptors for TGF-βs are found on nearly all cell types, but the nature of the biological response to TGF-βs varies with cell type. Exposure of epithelial cells to TGF-βs leads to inhibition of growth, induction of extracellular matrix protein formation, modulation of proteolysis, and stimulation of cell migration *(42,43)*. To initiate the signaling of these responses, TGF-β binds directly to the TGF-β type II receptor (TGFβRII) that is a constitutive active kinase, after which the TGF-β type I receptor (TGFβRI) is recruited into the complex *(47)*. The TGFβRII in the complex phosphorylates the GS domain of TGFβRI and then leads to propagation of further downstream signals. Mutational analyses altering serine and threonine residues in the TGFβRI GS domain have indicated that the phosphorylation by TGFβRII is indispensable for TGF-β signaling, although its signaling activity does not appear to depend on the phosphorylation of any particular serine or threonine residue in the TTSGSGSG sequence of the GS domain *(47,48)*.

Patel and colleagues *(10)* have first reported that polyamine depletion activates expression of the *TGF-β* gene in IECs. Administration of DFMO not only depletes intracellular polyamines, but also significantly increases the mRNA levels of TGF-β in IEC-6 cells. Increased TGF-β mRNA in DFMO-treated cells is paralleled by an increase in TGF-β content. To determine the mechanisms by which polyamine depletion induces the *TGF-β* gene expression, it has been shown that depletion of cellular polyamines by DFMO has no effect on the rate of *TGF-β* gene transcription as measured by a nuclear run-on assay, but dramatically increases half-life of mRNA for TGF-β. In control cells, the mRNA levels for TGF-β decline rapidly after inhibition of RNA syn-

thesis, with a half-life of 65 min. In polyamine-deficient cells, however, the stability of TGF-b mRNA is dramatically increased. TGF-b mRNA from cells exposed to DFMO for 6 d decreases at a slower rate, with a half-life of longer than 16 h. The increased stability of TGF-b mRNA in DFMO-treated cells is prevented when spermidine is given with DFMO. These results clearly indicate that polyamines affect the *TGF-b* gene posttranscriptionally, not transcriptionally, and that polyamine depletion induces the activation of *TGF-b* gene by increasing TGF-b mRNA stability.

5.2. Increased Expression of the TGF-b *Gene Contributes to Growth Inhibition in Polyamine-Deficient Cells*

Activation of *TGF-b* gene expression is involved in the process of growth inhibition after polyamine depletion *(10)*. In normal IECs, increased synthesis of TGF-b in polyamine-deficient cells is associated with a decrease in cell proliferation. Exogenous spermidine given with DFMO prevents the increased expression of the *TGF-b* gene and returns cell growth to near normal. TGF-b added to the culture medium also decreases the rate of total cellular DNA synthesis and final cellular number in normal and polyamine-deficient IEC-6 cells. Interestingly, polyamine-deficient cells are much more sensitive to exogenous TGF-b than normal IEC-6 cells (without DFMO). Furthermore, growth inhibition caused by polyamine depletion is partially but significantly blocked by addition on immunoneutralizing anti-TGF-b antibody.

In this immunoneutralizing study, cells were grown in the presence of DFMO for 4 d, and then the specific antibody against TGF-b at different concentrations was added to the medium containing DFMO. DNA synthesis and cell number were measured after incubation with the antibody for 48 h. Administration of anti-TGF-b antibody resulted in a significant induction of DNA synthesis and final cell number in polyamine-deficient cells. The rate of [^{3}H]-thymidine incorporation and cell number in the DFMO-treated cells were increased by approx 70 and 60%, respectively, when the antibody at the concentration of 20 mg/mL was given for 48 h. This antibody also has the ability to immunoneutralize exogenous TGF-b added to the culture medium in control or polyamine-deficient cells. On the other hand, heat-inactivated anti-TGF-b antibody has no additional effects on cell growth, regardless of the presence or absence of exogenous TGF-b. These results strongly suggest that increased expression of the *TGF-b* gene plays an important role in growth inhibition after polyamine depletion.

5.3. Expression of the TGF-b Receptor *Gene and Sensitivity to Exogenous TGF-b*

Polyamine-deficient cells are more sensitive to growth inhibition when they are exposed to exogenous TGF-b *(10)*. Because IECs produce both TGF-b receptors and ligands *(49,50)*, it is possible that the regulation of cellular responsiveness relies on the production of active TGF-b and its presentation to signaling receptors. Rao and colleagues *(11)* have further reported that depletion of cellular polyamines increases levels of the TGF-bRI mRNA and protein, but has no effect on the TGF-bRII receptor expression in IEC-6 cells. The induced TGF-bRI expression after polyamine depletion

is associated with an increased sensitivity to growth inhibition induced by exogenous TGF-b, but not by somatostatin. The increased TGF-bRI expression and induced sensitivity to TGF-b-mediated growth inhibition appear to be specific to polyamine depletion, because laminin-induced growth inhibition does not increase TGF-bRI expression and thus fails to affect cellular responsiveness to exogenous TGF-b. Furthermore, decreasing TGF-bRI expression by treatment with retinoic acid not only decreases TGF-b-mediated growth inhibition in normal cells, but also prevents the increased sensitivity to exogenous TGF-b in polyamine-deficient cells. These findings indicate that expression of the *TGF-bRI* gene is highly regulated by cellular polyamines, and that increased TGF-bRI expression following polyamine depletion plays an important role in the increased sensitivity to TGF-b-mediated growth inhibition.

5.4. Smads are the Downstream Intracellular Effectors of Activated TGF-b/TGF-b Receptors After Polyamine Depletion

The Smad proteins are a family of transcriptional activators that are critical for transmitting the TGF-b superfamily signals from the cell surface to the nucleus *(51,52)*. Based on distinct functions, Smads are grouped into three classes: the receptor-regulated Smads (R-Smads), Smad2, and Smad3; the common Smad (co-Smad), Smad4; and the inhibitory Smads (I-Smads), Smad6, and Smad7 *(53–55)*. All TGF-b family members, including TGF-bs, activins, and bone morphogenetic proteins, use TGF-bRI and TGF-bRII receptors in a variety of cell types *(53,56)*. After ligand binding, the activated TGF-bRII kinase phosphorylates the TGF-bRI receptor, which subsequently phosphorylates the R-Smads on a C-terminal SSXS motif. This induces dissociation of the R-Smad from the receptor, stimulates the assembly of a heteromeric complex between the phosphorylated R-Smads and Smad4, and results in the nuclear accumulation of this heteromeric Smad3/Smad4 complex *(55–57)*. In the nucleus, Smads bind to a specific DNA site (GTCTAGAC) and cooperate with various transcription factors in regulating target gene expression *(58,59)*.

We have recently demonstrated that Smad proteins are involved in the process mediating functions of cellular polyamines in IECs *(60)*. Exposure of IEC-6 cells to DFMO for 4, 6, and 8 d significantly increased levels of both Smad3 and Smad4 proteins and induced their nuclear translocation. Smad3 is shown to be highly expressed in IECs and activation of this R-Smad is ligand-specific. It is not surprising that polyamine depletion increases Smad3 protein and enhances its nuclear translocation because decreased levels of cellular polyamines are known to stimulate expression of TGF-b/TGF-b receptors in IEC-6 cells *(10,11)*. Activated Smad3 results primarily from the increase in expression of TGF-b/TGF-b receptors in polyamine-deficient cells because inhibition of TGF-b by treatment with either immunoneutralizing anti-TGF-b antibody or TGF-b antisense oligomers prevents the increased Smad activation in the absence of cellular polyamines.

Polyamine depletion also induces Smad4 nuclear translocation in IEC-6 cells. Smad4 functions as a common mediator for all R-Smads, and forms heteromeric complexes with Smad3 after ligand activation *(52,53)*. The observed change in Smad4 in polyamine-deficient cells, however, seems to be a secondary response to the activation

of Smad3. In support of this possibility, treatment with exogenous TGF-b did not alter levels of Smad4 protein in normal IEC-6 cells (without DFMO), although it significantly increased Smad3 expression. Furthermore, exposure to immunoneutralizing anti-TGF-b antibody or TGF-b antisense oligomers did not prevent the increased levels of Smad4 protein in polyamine-deficient cells. The other possibility also exists that polyamine depletion induces Smad4 expression through a mechanism independent from the activated Smad3 in IECs. In addition, this increased Smad expression and nuclear translocation in the DFMO-treated cells are specifically related to polyamine depletion rather than to a nonspecific effect of DFMO, because the stimulatory effect of this compound on Smads was completely prevented by the addition of exogenous spermidine.

Importantly, polyamine depletion-induced Smad activity is associated with a significant increase in transcriptional activation of Smad-driven promoters. Using the electrophoretic mobility shift method and luciferase reporter assays, we have demonstrated that polyamine depletion significantly increases Smad sequence-specific DNA binding and induces luciferase reporter activity of Smad-dependent promoters *(60)*. Our results are consistent with others that indicated that induced nuclear Smad proteins mediate transcription of Smad target genes in a variety of cell types *(56,57)*. Our studies also show that increased transcriptional activation after polyamine depletion is primarily owing to the function of Smad3/Smad4 heteromeric complexes because ectopic expression of a dominant negative mutant, Smad4, prevented the increased Smad transcriptional activation in polyamine-deficient cells. Recently, Smad DNA-binding sites have been identified in various gene promoters, including *PAI*-1, *JunB*, *c-Jun*, and *p21* by different approaches. In general, these sequences reported by different groups are essentially identical; regardless of whether one defines a Smad-binding site as the palindrome AGACGTCT, as the CAGA box, or as repeats of GACA, all identified sites contain the Smad box, 5¢-GTCT-3¢, or its reverse complement, 5¢-AGAC-3¢. Our results clearly indicate that induced Smad3/Smad4 heteromeric complexes in polyamine-deficient cells are able to bind to this specific DNA site, suggesting that Smads mediate transcriptional activation, probably through a direct interaction with Smad-binding element (SBE) sites in target promoters.

Combining these findings with our previous studies *(10,11,60)*, it has been shown that activation of the TGF-b/Smad signaling pathway plays a role in the inhibition of IEC proliferation after polyamine depletion (Fig. 6). Polyamines negatively regulate posttranscription of the *TGF-b* gene. Decreased cellular polyamines stabilize TGF-b mRNAs, increase TGF-b synthesis, and cause the activation of TGF-b receptors. Smads are the downstream intracellular effectors of activated TGF-b receptors after polyamine depletion. The resultant activation of TGF-b receptors induces Smad3/Smad4 levels and stimulates Smad nuclear translocation, resulting in the accumulation of nuclear Smad3/Smad4 complexes. In the nucleus, Smads bind to the specific DNA site and cooperate with their DNA-binding partners, such as JunD/AP-1, to activate or repress transcription of specific target genes, thus leading to the inhibition

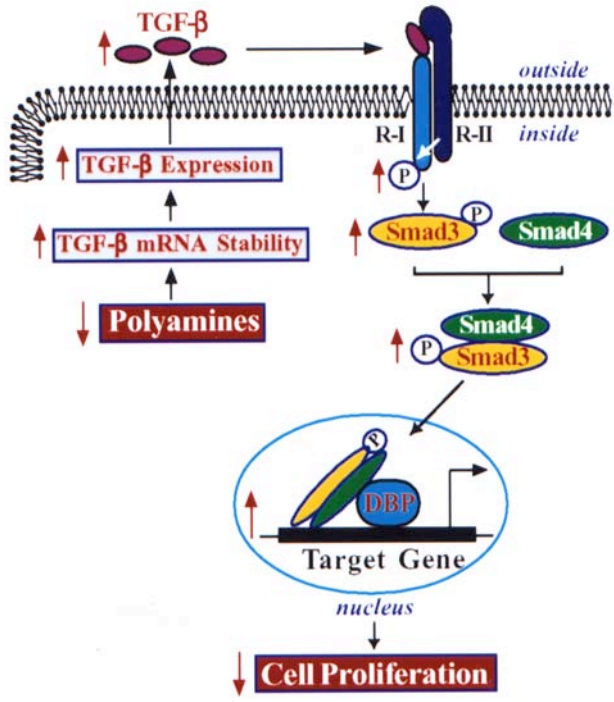

Fig. 6. Schematic diagram depicting the proposed role of the TGF-b/Smad signaling pathway in the inhibition of cell proliferation after polyamine depletion. In this model, polyamines are the negative regulators for expression of the *TGF-b* gene, whereas Smad proteins are the downstream intracellular effectors of activated TGF-b receptors. Decreased levels of cellular polyamines increase expression of TGF-b through stabilization of TGF-b mRNA, enhance the release of TGF-b, and subsequently phosphorylate TGF-b type II receptor (R-II). The phosphorylated R-II activates TGF-b type I receptor (R-I), induces the formation of Smad3/Smad4 heteromeric complexes, and stimulates their nuclear translocation. The activated Smads in the nucleus bind to the specific DNA site and cooperate with Smad DNA-binding partners, such as some AP-1 proteins, to activate or repress transcription of specific target genes, thus leading to

of IEC proliferation. In contrast, increased polyamines downregulate the TGF-b/Smad signaling pathway and enhance IEC proliferation.

6. Summary and Conclusions

The experimental data summarized in this chapter provide evidence supporting the hypothesis that polyamine depletion causes inhibition of cell proliferation primarily through the activation of specific cellular signaling pathways, especially increased expression of growth-inhibiting genes. To date, three cellular signaling pathways that have been well characterized and greatly contribute to growth arrest after polyamine depletion are: (1) p53/p21 signaling; (2) JunD/AP-1 signaling; and (3) TGF-b/Smad

signaling. All three of these signals belong to the category of growth-inhibiting factors that are encoded by growth-inhibiting genes. The activation of these genes results in growth inhibition through different downstream targets in a variety of cell types. Because negative growth control, including growth arrest and apoptosis, is critical for the control of appropriate cell numbers in normal tissues, alterations in any part of the equation affect tissue homeostasis, leading to, for example, mucosal atrophy or loss of epithelial integrity. Therefore, studies to define the exact roles and mechanisms of cellular polyamines in expression of these growth-inhibiting genes are important and have potential clinical application.

Interestingly, polyamines modulate expression of growth-inhibiting genes such as *p53*, *junD*, and *TFG-b* at the posttranscriptional, but not transcriptional, level. Increased levels of cellular polyamines inhibit expression of these growth-inhibiting genes by increasing degradation of their mRNAs and proteins, whereas polyamine depletion increases levels of these growth-inhibiting factors by stabilizing their mRNAs and proteins. Although the exact mechanisms by which polyamines regulate stability of these growth-inhibiting proteins remain unclear, it has been shown that induced expression of *p53*, *junD*, and *TFG-b* genes results in G_1 phase growth arrest after polyamine depletion. However, many critical issues regarding growth arrest induced by polyamine depletion remain to be demonstrated. Studies to define the molecular process responsible for posttranscriptional regulation of growth-inhibiting genes by polyamines and how polyamine depletion-induced growth-inhibiting proteins interact with their downstream signaling targets are needed and will lead to a better understanding of biological functions of cellular polyamines and the mechanism of polyamine depletion-induced growth arrest under various physiological and pathological conditions.

References

1. Tabor, C. W. (1984) Polyamines. *Ann. Rev. Biochem.* **53,** 749–790.
2. Gerner, E. W. and Meyskens, F. L. (2004) Polyamines and cancer: old molecules, new understanding. *Nat. Rev.* **4,** 781–792.
3. Luk, G. D., Marton, L. J., and Baylin, S. B. (1980) Ornithine decarboxylase is important in intestinal mucosal maturation and recovery from injury in rats. *Science* **210,** 195–198.
4. Celano, P., Baylin, S. B., and Casero, R. A. (1989) Polyamines differentially modulate the transcription of growth-associated genes in human colon carcinoma cells. *J. Biol. Chem.* **264,** 8922–8927.
5. Wang, J. Y., McCormack, S. A., Viar, M. J., et al. (1993) Decreased expression of protooncogenes *c-fos*, *c-myc*, and *c-jun* following polyamine depletion in IEC-6 cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* **265,** G331–G338.
6. Li, L., Li, J., Rao, J. N., Li, M.-L., Bass, B. L., and Wang, J. Y. (1999) Inhibition of polyamine synthesis induces p53 gene expression but not apoptosis. *Am. J. Physiol. Cell. Physiol.* **276,** C946–C954.
7. Kramer, D. L., Vujcic, S., Diegelman, P., et al. (1999) Polyamine analogue induction of the p53-$p21^{WAF1/CIP1}$-Rb pathway and G1 arrest in human melanoma cells. *Can. Res.* **59,** 1278–1286.

8. Li, L., Liu, L., Rao, J. N., et al. (2002) JunD stabilization results in inhibition of normal intestinal epithelial cell growth through p21 after polyamine depletion. *Gastroentrology* **123,** 764–779.
9. Patel, A. R. and Wang, J. Y. (1999) Polyamine depletion is associated with an increase in JunD/AP-1 activity in small intestinal crypt cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* **276,** G441–G450.
10. Patel, A. R., Li, J., Bass, B. L., and Wang, J. Y. (1998) Expression of the transforming growth factor-b gene during growth inhibition following polyamine depletion. *Am. J. Physiol. Cell. Physiol.* **275,** C590–C598.
11. Rao, J. N., Li, L., Bass, B. L., and Wang, J. Y. (2000) Expression of the TGF-b receptor gene and sensitivity to growth inhibition following polyamine depletion. *Am. J. Physiol. Cell. Physiol.* **279,** C1034–C1044.
12. Patel, A. R. and Wang, J. Y. (1997) Polyamines modulate transcription but not posttranscription of *c-myc* and *c-jun* in IEC-6 cells. *Am. J. Physiol.* **273,** C1020–C1029.
13. Liu, L., Li, L., Rao, N. J., et al. (2005) Polyamine-modulated expression of *c-myc* plays a critical role in stimulation of normal intestinal epithelial cell proliferation. *Am. J. Physiol. Cell. Physiol.* **288,** C89–C99.
14. Wang, J. Y. and Johnson, L. R. (1994) Expression of protooncogenes *c-fos* and *c-myc* in healing of gastric mucosal stress ulcers. *Am. J. Physiol. Gastrointest. Liver Physiol.* **266,** G878–G886.
15. Li, L., Rao, J. N., Bass, B. L., and Wang, J. Y. (2001) NF-kB activation and susceptibility to apoptosis following polyamine depletion in intestinal epithelial cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* **280,** G992–G1004.
16. Slee, E. A., O'Connor, D. J., and Lu, X. (2004) To die or not die: how does p53 decide? *Oncogene* **23,** 2809–2818.
17. Appella, E. and Anderson, C. W. (2001) Post-transcriptional modifications and activation of p53 by genotoxic stresses. *Eur. J. Biochem.* **268,** 2764–2772.
18. Kurki, S., Peltonen, K., and Laiho, M. (2004) Nucleophosmin, HDM2 and p53: players in UV damage incited nucleolar stress response. *Cell Cycle* **3,** 976–979.
19. Fang, S., Jensen, J. P., Ludwig, R. L., Vousden, K. H., and Weissman, A. M. (2000) Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53. *J. Biol. Chem.* **275,** 8945–8951.
20. Li, L., Rao, J. N., Li, J., Patel, A. R., Bass, B. L., and Wang, J. Y. (2001) Polyamine depletion stabilizes p53 resulting in inhibition of normal intestinal epithelial cell proliferation. *Am. J. Physiol. Cell. Physiol.* **281,** C941–C953.
21. Mercer WE, Shields MT, Amin M, et al. (1990) Negative growth regulation in glioblastoma tumor cell line that conditionally expresses human wild-type p53. *Proc. Natl. Acad. Sci. USA* **87,** 6166–6170.
22. Kramer, D. L., Chang, B. D., Chen, Y., et al. (2001) Polyamine depletion in human melanoma cells leads to G1 arrest associated with induction of $p21^{WAF1/CIP1/SDI1}$, changes in the expression of p21-regulated genes, and a senescence-like phenotype. *Can. Res.* **61,** 7754–7762.
23. Ray, R. M., Zimmerman, B. J., McCormack, S. A., Patel, T. B., and Johnson, L. R. (1999) Polyamine depletion arrests cell cycle and induces inhibitors $p21^{Waf1/Cip1}$, $p27^{Kip1}$, and p53 in IEC-6 cells. *Am. J. Physiol. Cell. Physiol.* **276,** C684–C691.
24. Pfeffer, L. M., Yang, C. H., Pfeffer, S. R., Murti, A., McCormack, S. A., and Johnson, L. R. (2000) Inhibition of ornithine decarboxylase induces STAT3 tyrosine phosphorylation and DNA binding in IEC-6 cells. *Am. J. Physiol. Cell. Physiol.* **278,** C331–C335.

25. Maiguel, D. A., Jones, L., Chakravarty, D., Yang, C., and Carrier, F. (2004) Nucleophosmin sets a threshold for p53 response to UV radiation. *Mol. Cell. Biol.* **24,** 3703–3711.
26. Wu, M. H. and Yung, B. Y. M. (2002) UV stimulation of nucleophosmin/B23 expression is an immediate-early gene response induced by damage DNA. *J. Biol. Chem.* **277,** 48,234–48,240.
27. You, Y., Chen, C.-Y., and Shyu, A. B. (1992) U-rich sequence-binding protein (URBPs) interacting with a 20-uncleotide U-rich sequence in the 3¢ untranslated region of *c-fos* mRNA may be involved in the first step of *c-fos* mRNA degradation. *Mol. Cell. Biol.* **12,** 2931–2940.
28. Paulding, W. R. and Czyzk-Krzeska, M. F. (1999) Regulation of tyrosine hydroxylase mRNA stability by protein-binding, pyrimidine-Reich sequence in the 3¢-untranslated region. *J. Biol. Chem.* **274,** 2532–2538.
29. Shih, S.-C. and Claffey, K. P. (1999) Regulation of human vascular endothelial growth factor mRNA stability in hypoxia by heterogeneous nuclear ribonucleoprotein L. *J. Biol. Chem.* **274,** 1359–1365.
30. Schmidt-Zachmann, M. S., Hugle-Dorr, B., and Franke, W. W. (1987) A constitutive nucleolar protein identified as a member of the nucleoplasmin family. *EMBO J.* **6,** 1881–1890.
31. Chan, W. Y., Liu, Q. R., Borjigin, J., et al. (1989) Characterization of the cDNA encoding human nucleophosmin and studies of its role in normal and abnormal growth. *Biochemistry* **28,** 1033–1039.
32. Zou, T., Rao, J. N., Liu, L., et al. (2005) Polyamine depletion induces nucleophosmin modulating stability and transcriptional activity of p53 in intestinal epithelial cells. *Am. J. Physiol. Cell. Physiol.* **289,** C686–C696.
33. Zhang, H. F., Rao, J. N., Guo, X., et al. (2004) Akt kinase activation blocks apoptosis in intestinal epithelial cells by inhibiting caspase-3 after polyamine depletion. *J. Biol. Chem.* **279,** 22,539–22,547.
34. Wang, J. Y., McCormark, S. A., Viar, M. J., and Johnson, L. R. (1991) Stimulation of proximal small intestinal mucosal growth by luminal polyamines. *Am. J. Physiol. Gastrointest. Liver Physiol.* **261,** G504–G511.
35. Hall, P. A., Coates, P. J., Ansari, B., and Hopwood, D. (1994) Regulation of cell number in the mammalian gastrointestinal tract—the importance of apoptosis. *J. Cell. Sci.* **107,** 3569–3577.
36. Potten, C. S. and Loeffler, M. (1990) Stem cells: attributes, cycles, spirals, pitfalls, and uncertainties-lessons for and from the crypt. *Development* **110,** 1001–1020.
37. Hirai, S. I., Ryseck, R. P., Mechta, F., Bravo, K., and Yaniv, M. (1989) Characterization of *junD*: a new member of the jun proto-oncogene family. *EMBO J.* **8,** 1433–1439.
38. Ryder, K., Lanahan, A., Perez-Albuerne, E., and Nathans, D. (1989) JunD: a third member of the jun gene family. *Proc. Natl. Acad. Sci. USA* **86,** 1500–1503.
39. Angel, P., Allegretto, E. A., Okino, S. T., et al. (1988) Oncogene Jun encodes a sequence-specific transactivator similar to AP-1. *Nature* **332,** 166–171.
40. Nakabeppu, Y., Ryder, K., and Nathans, D. (1988) DNA binding activities of three murine Jun proteins: stimulation by Fos. *Cell* **55,** 907–915.
41. Pfarr, C. M., Mechta, F., Spyrou, G., Lallemand, D., Carillo, S., and Yaniv, M. (1994) Mouse JunD negatively regulates fibroblast growth and antagonizes transformation by ras. *Cell* **76,** 747–760.
42. Barnard, J. A., Lyons, R. M., and Moses, H. L. (1990) The cell biology of transforming growth factor b. *Biochim. Biophys. Acta.* **1032,** 79–87.
43. Barnard, J. A., Warwick, G. J., and Gold, L. I. (1993) Localization of transforming growth factor-b isoforms in the normal murine small intestine and colon. *Gastroenterology* **105,** 67–73.

44. Franzen, P., Dijke, P. T., Ichijo, H., et al. (1993) Cloning of a TGFb type I receptor that forms a heteromeric complex with the TGFb type II receptor. *Cell* **75,** 681–692.
45. Massague J. (1992) Receptors for the TGF-b family. *Cell* **69,** 1067–1070.
46. Sarkar, D. K., Pastorcic, M., De, A., Engel, M., Moses, H., and Ghasemzadeh, B. (1998) Role of transforming growth factor (TGF)-b type I and TGF-b type II receptors in the TGF-b1-regulated gene expression in pituitary prolactin-secreting lactotropes. *Endocrinology* **139,** 3620–3628.
47. Wrana, J. L., Attisano, L., Wieser, R., Ventura, F., and Massague, J. (1994) Mechanism of activation of the TGF-b receptor. *Nature* **370,** 341–347.
48. Wieser, R., Wrana, J. L., and Massague, J. (1995) GS domain mutations that constitutively activate TbR-I, the downstream signaling component in the TGF-b receptor complex. *EMBO J.* **14,** 2199–2208.
49. Barnard, J. A., Beauchamp, R. D., Coffey, R. J., and Moses, H. L. (1989) Regulation of intestinal epithelial cells growth by transforming growth factor type b. *Proc. Natl. Acad. Sci. USA* **86,** 1578–1582.
50. Suemori, S., Ciacci, C., and Podolsky, D. K. (1991) Regulation of transforming growth factor expression in rat intestinal epithelial cell lines. *J. Clin. Invest.* **87,** 2216–2221.
51. Attisano, L. and Wrana, J. L. (2000) Smads as transcriptional co-modulators. *Curr. Opin. Cell. Biol.* **12,** 235–243.
52. Zawel, L., Dai, J. L., Buckhaults, P., et al. (1998) Human Smad3 and Smad4 are sequence specific transcription activators. *Mol. Cell.* **1,** 611–617.
53. Itoh, S., Itoh, F., Goumans, M. J., and Dijke, P. T. (2000) Signaling of transforming growth factor-b family members through Smad proteins. *Eur. J. Biochem.* **267,** 6954–6967.
54. Liberati, N. T., Moniwa, M., Borton, A. J., Davie, J. R., and Wang, X. F. (2001) An essential role for mad homology domain 1 in the association of Smad3 with histone deacetylase activity. *J. Biol. Chem.* **276,** 22,595–22,603.
55. Massague, J. and Wotton, D. (2000) Transcriptional control by the TGF-b/Smad signaling system. *EMBO J.* **19,** 1745–1754.
56. Heldin, C. H., Miyazono, K., and Dijke, P. T. (1997) TGF-b signaling from cell membrane to nucleus through SMAD proteins. *Nature* **390,** 465–471.
57. Dennler, S., Itoh, S., Vivien, D., Dijke, P. T., Huet, S., and Gauthier, J. M. (1998) Direct binding of Smad3 and Smad4 to critical TGF-b-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. *EMBO J.* **17,** 3091–3100.
58. Dijke, P. T., Goumans, M. J., Itoh, F., and Itoh, S. (2002) Regulation of cell proliferation by Smad proteins. *J. Cell. Physiol.* **191,** 1–16.
59. Wong, C., Rougier-Chapman, E. M., Frederick, J. P., et al. (1999) Smad3-Smad4 and AP-1 complexes synergize in transcriptional activation of the c-Jun promoter by transforming growth factor b. *Mol. Cell. Biol.* **19,** 1821–1830.
60. Liu, L., Santora, R., Rao, J. N., et al. (2003) Activation of TGF-b/Smad signaling pathway following polyamine depletion in intestinal epithelial cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* **285,** G1056–G1067.

5

Role of Polyamines in the Regulation of Chromatin Acetylation

Cheryl A. Hobbs and Susan K. Gilmour

1. Introduction

Changes in chromatin structure can affect gene transcription, cell proliferation, and differentiation *(1)*. The structural remodeling of chromatin associated with gene expression is mediated in part by the coordinated targeting of various chromatin modifying enzymes to gene regulatory regions via recruitment by transcription factors and accessory proteins *(2)*. The dynamic interplay between various classes of enzymes that acetylate/deacetylate, phosphorylate/dephosphorylate, and methylate/demethylate proteins determines the pattern of chemically modified amino acid residues in the N-terminal tail domains of the core histones that comprise nucleosomes. The specific pattern of histone modification, or "histone code," is then interpreted by proteins that selectively bind to modified residues, providing a platform for the protein machinery responsible for establishing the chromatin into a transcriptionally silent or activated state *(3,4)*.

Interestingly, correlating increases in histone acetylation and polyamines have been associated with cellular proliferation *(5)*, suggesting that the small cationic polyamines may be involved in chromatin remodeling and transcriptional regulation. In fact, changes in intracellular levels of polyamines do lead to altered gene expression in cells. For example, the transcriptional activity of a variety of mammalian and viral gene promoters, as measured by reporter gene activity, is enhanced in transfected epidermal cells having elevated levels of ornithine decarboxylase (ODC) and polyamines *(6)*. Furthermore, depletion of polyamines by treatment with the specific inhibitor of ODC enzymatic activity, α-difluoromethylornithine (DFMO), was shown to result in downregulation of *c-fos*, *c-myc*, and *c-jun* expression in colon cells, which could be reversed by the addition of spermidine *(7,8)*. However, although spermidine addition to nuclei from colorectal cells increased the transcription of *c-fos* and *c-myc*, not all genes examined were affected *(7)*. Moreover, expression profiling studies indicate that polyamines upregulate some genes, whereas they downregulate others *(9)*. These studies

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suggest that polyamines probably do not induce global effects on transcription, but may stimulate or repress different subsets of genes relying on common transcription factors or regulatory mechanisms. This chapter will focus on possible mechanisms by which polyamines may influence nucleosome acetylation status and chromatin structure, thus affecting transcriptional regulation.

1.1. Effects of Polyamines on Chromatin Structure

Because of their cationic nature and strong affinity for DNA, it has long been thought that polyamines contribute to overall chromosome conformation, potentially influencing gene transcription. Indeed, biophysical and biochemical studies have shown that the highly positively charged polyamines bind strongly to DNA and affect its conformational state *(10,11)*. Use of photoaffinity polyamine analogs has revealed that polyamines bind to specific locations and change the helical twist of DNA in nucleosomes *(11)*. Spermidine and spermine facilitate the condensation of chromatin fragments in vitro *(12)*, and studies using polyamine-depleted cells have suggested a role for polyamines in chromatin condensation *(13–15)*. However, results of these early cell-based studies need to be reinterpreted in light of subsequent experiments demonstrating that polyamine depletion in Chinese hamster ovary cells deficient in ODC activity leads to accumulation of cells in the S phase of the cell cycle, when the chromatin exists in a noncompacted state *(16)*. This latter study also suggested that, although polyamines may have some stabilizing effect on chromatin structure, they do not play a critical role in the control of chromatin condensation throughout the cell cycle, as evaluated by general susceptibility to micrococcal nuclease. However, assays based on overall nuclease digestion of total chromatin may not be sufficiently sensitive to detect the subtle changes in nucleosome structure promoted by nonglobal acetylation or deacetylation of nucleosomal histones, which may be highly localized to one or two nucleosomes within specific gene promoters *(17)*.

Spermidine has been shown to efficiently induce the intermolecular association of nucleosomal arrays in vitro; interestingly, acetylation of histones decreases the effectiveness of spermidine to promote these fiber–fiber interactions *(18)*. Depletion of cellular polyamines in *gcn5* mutant strains of *Saccharomyces cerevisiae* partially restored expression of genes dependent on the transcriptional coactivator/histone acetyltransferase (HAT) protein, GCN5 *(18)*. Together, these results suggest the interesting possibility that histone acetylation and polyamines have opposing effects on chromatin fiber condensation in vivo, and that polyamines might be more likely to interact with domains of deacetylated chromatin to form a higher order chromatin structure that is incompatible with transcription. However, polyamine depletion in the *spe*$^-$ mutant yeast strains used in these genetic studies resulted in impaired growth and eventual arrest in the G_1 stage of the cell cycle *(18)*. Thus the partial restoration of defects in GCN5-dependent transcription resulting from prolonged deprivation of cellular polyamines may be specifically related to an accumulation of cells in G_1. Studies involving mammalian cells frequently rely on the ODC inhibitor, DFMO, to deplete cells of polyamines. Whether achieved through genetic manipulation or chemical inhibition, it is important to note that polyamine depletion may not necessarily produce

opposite effects on chromatin structure than those resulting from increased levels of intracellular polyamines. In any case, that polyamine depletion partially alleviated defects in GCN5-dependent gene expression, yet didn't lead to global effects on transcription *(18)*, is further evidence that polyamines influence the expression of only a subset of genes.

Although valuable information can be gleaned from cells in culture, very often, biological effects differ in animal or human tissue. An excellent animal model system that can be used to evaluate the effects of increased polyamine biosynthesis in an epithelial tissue type is the K6/ODC transgenic mouse. Overexpression of ODC enzyme activity is targeted to the outer root sheath cells of hair follicles in the skin of K6/ODC mice *(19)*. Cellular proliferation is increased in epithelial cells that overexpress ODC, causing the loss of hair and the formation of follicular cysts in the dermis. Although overexpression of ODC in the skin of K6/ODC mice does not lead to tumor formation, ODC overexpression cooperates with a mutated *H-ras* to produce spontaneous skin tumors in ODC/Ras double transgenic mice *(20)*. We have capitalized on the ODC transgenic mouse models to provide in vivo evidence of polyamine effects on chromatin structure.

Dissociation of histones from chromatin from ODC transgenic mouse skin and tumors was found to be dramatically reduced relative to normal littermate skin; this phenomenon is reversible on treatment with the specific inhibitor of ODC enzymatic activity, DFMO *(21)*. One possible explanation for this observation is that polyamines bind directly to chromosomal DNA to alter its conformation into a more compacted structure that leads to entrapment of nucleosomes. Another possible scenario is that polyamines either directly or indirectly affect the histones themselves, or protein complexes that interact with histones, thus strengthening the tethering of nucleosomes to DNA. This might occur as a result of polyamine modulation of the enzymatic activities or the amount available of chromatin remodeling proteins. Indeed, studies reviewed later in this chapter demonstrate that polyamines can exert both positive and negative influence on histone acetylation and modulate the intrinsic activities of HAT and histone deacetylase (HDAC) enzymes, which likely have localized consequences for chromatin structure. Additionally, polyamines may influence the phosphorylation or methylation status of histones and other chromatin proteins, resulting in an altered affinity for DNA or other components of chromatin protein complexes. Interestingly, there appears to be selective effects of polyamines on specific chromatin protein complexes because heterochromatin protein 1β (HP-1β) and nucleophosmin/B23 are also more tightly associated with chromatin from K6/ODC skin, whereas the release of other nonhistone chromatin proteins, such as Lamin B, HDAC-1, HMGB, HMGN2, and HMGA1, is not affected by high levels of cellular polyamines *(21)*. Global acetylation and methylation of histones is not significantly affected in transgenic skin overexpressing ODC compared with normal littermate skin *(21)*. Thus the altered chromatin structure in K6/ODC transgenic mouse skin does not appear to generally restrict recruitment of histone acetylating, deacetylating, or methylating enzymes. Combined, these results suggest that the constraints imposed on chromatin by polyamines are more complex than can be attributed simply to DNA compaction.

Polyamine-induced chromatin alterations may have interesting long-term effects that promote carcinogenesis. It has been proposed that chromatin structure and covalent modifications of histones may imprint an epigenetic code on transcription *(3,22,23)*. Results of our studies suggest that HP-1β is somehow hindered from dissociating from chromatin in ODC transgenic mouse skin. The HP-1 protein has been implicated in the formation of heterochromatin, gene silencing, and in the stable inheritance of heterochromatin assembly *(24)*. Moreover, a novel role for HP-1 in the transcriptional repression of euchromatic genes by the retinoblastoma protein (Rb) has been described *(25)*. It is possible that the tighter association of HP-1 in chromatin resulting from the elevated levels of polyamines in ODC mouse skin may lead to the transfer of epigenetic "marks" and the subsequent propagation of an inappropriately silenced state to newly synthesized strands of DNA. Elevated levels of polyamines are an essential component of the promotional stage of tumorigenesis *(26)*. Conceivably, the development of tumors in tissues that harbor dormant mutations may be promoted via polyamine-induced changes in chromatin structure that continue to be propagated through many cell mitoses.

1.2. Effects of Elevated Levels of ODC and Polyamines on Chromatin-Modifying Enzymes

Histones function as transcriptional modulators as a result of sophisticated molecular mechanisms that regulate their modification *(1–4,22)*. The transcriptional status of a gene hinges largely on the acetylation status of the nucleosomal histones bound to the gene. Addition of acetyl groups to lysines in the N-terminal tail domains of the core histones, catalyzed by HAT enzymes, destabilizes nucleosomes, allowing regulatory factors access to recognition elements *(27)*. Conversely, deacetylation of histones by HDAC enzymes typically stabilizes the repressed state.

Early experiments suggested that polyamines may directly influence HAT or HDAC enzyme activity. Both spermidine and spermine were shown to stimulate HAT activity *(28,29)* and to inhibit HDAC activity in cell-free systems *(30,31)*. Moreover, an increase in polyamines was correlated with enhanced RNA synthesis, tissue growth, and histone acetylation during the early stages of rabbit myocardial hypertrophy *(5)*. However, the observation that low levels of exogenous polyamines release chromatin-bound deacetylase enzyme *(30)* indicates that polyamines may also modulate interactions between HAT/HDAC proteins and associating cofactors, DNA binding proteins, or components of the RNA polymerase II machinery. Unfortunately, technical problems inherent to cell-free assays and cell culture studies complicate their interpretation. For example, it is difficult to form conclusions about the natural interaction of polyamines with chromatin components after a chromatin fiber has lost its normal compact morphology as occurs on its isolation from the nucleus. Detergent permeabilization of cell membranes to permit use of molecules to monitor chromatin structure or to isolate nuclei also damages the fibers *(32)*. Moreover, uptake of exogenous polyamines does not necessarily simulate the normal distribution of naturally synthesized polyamines. By using cultured cells and mice genetically engineered to overexpress ODC, the role of polyamines in modulating the function of HAT and HDAC enzymes can be assessed within the natural context of a proliferating cell or

mammalian tissue. Furthermore, the ODC/Ras double transgenic mouse serves as a valuable model for correlating processes that regulate histone acetylation with real-time tumor formation and regression. As discussed later in this chapter, we used such tools to show that elevated levels of polyamines alter histone acetylation and HAT and HDAC activities in proliferating cells, and in murine skin and tumors *(33,34)*. These studies provide the first evidence of polyamine-mediated effects on chromatin structure in mammals and, furthermore, suggest a mechanism by which aberrant polyamine biosynthesis contributes to cellular transformation.

2. Effects of Elevated Intracellular Levels of Polyamines on Cultured Mammalian Cells

Experiments relying on exogenous addition of polyamines to assay buffers or on the cellular uptake of polyamines added to cell culture medium do not always mimic the normal compartmentalization of polyamines synthesized naturally in the cell, or necessarily lead to the same biological outcome *(6,35)*. For this reason, we have used strategies relying on the manipulation of the natural polyamine biosynthetic pathway to examine the consequences of elevated intracellular polyamines on histone acetylation in proliferating cells. Mammalian cells were retrovirally engineered to overexpress ODC, labeled with [^{3}H] acetate, and acetylated histones examined by fluorography and Western analyses. Given the early work suggesting stimulatory effects of polyamines on HAT enzymes in crude extracts *(28,29)*, we were not surprised to find that elevated levels of ODC promote acetylation of histones in the several immortalized epidermal and fibroblast cell lines tested *(33)*. The ODC-mediated increase in acetylated histones was abrogated when cells were treated with DFMO, indicating that polyamines were specifically responsible for the observed effect on histone acetylation. Similar polyamine effects on histone acetylation were observed in G_2/M-synchronized and asynchronously growing cell populations. Because deacetylation of lysine residues in histone tails by HDAC enzymes typically occurs very rapidly, cells were always labeled in the presence of 10 m*M* sodium butyrate, an effective HDAC inhibitor. Given that deacetylase activity was efficiently blocked in these experiments, the increased acetylation observed in the ODC-overexpressing cells reflects a direct or indirect effect of polyamines specifically on HAT enzyme activity. Definitive assessment of the effects of polyamines on HDAC activity must await the development of specific, cell-permeable inhibitors of HAT enzymes.

The increase in acetylated histone H4 detected in cells constitutively overexpressing ODC was determined to be owing to an increase in hyperacetylated histone isoforms, rather than simply a greater overall level of monoacetylated histones *(33)*. Hyperacetylated histones are found in nucleosomes associated with transcriptionally active chromatin. Therefore, our observation that ODC overexpression cooperates with histone deacetylase inhibitors to further enhance reporter gene expression beyond that promoted by ODC or inhibitor treatment alone *(33)* might be explained by the proportionally greater amounts of the more highly acetylated histone isoforms in these cells. The increased abundance of mono- and hyperacetylated histones in ODC-overexpressing cells necessitates that some proportion of nucleosomes comprise histones having a

different combination of nonacetylated and acetylated lysine residues as compared with normal circumstances. These changes in the pattern of lysine acetylation may result in an altered binding affinity of nucleosomes for DNA, or altered interactions between neighboring nucleosomes, perhaps affecting nucleosomal positioning or access by factors critical for transcription and other cellular processes. In fact, highly specific patterns of acetylated lysine residues occur in histones that act as signals for the recruitment of proteins that facilitate the reconfiguration of chromatin associated with gene expression, DNA replication, repair, recombination, and differentiation. Thus the altered patterns of histone acetylation promoted by high intracellular concentrations of polyamines might be expected to cause aberrant function of one or more of these cellular programs. Indeed, inappropriate histone acetylation has been linked to both the loss of the differentiated phenotype and cell transformation *(36,37)*.

2.1. Effects of Increased Polyamine Biosynthesis in Mouse Skin

To examine the effects of elevated ODC and polyamines on histone acetylation in a more relevant physiological system in which epithelial cells can still both proliferate and differentiate, unlike the immortalized cell lines studied in the earlier experiments, primary keratinocytes were isolated and cultured from K6/ODC transgenic and normal littermate skin *(34)*. After labeling the keratinocytes with [^{3}H]acetic acid in the presence of sodium butyrate, histones were isolated and examined by fluorography and by immunoblot analyses. In contrast to what was observed in immortalized cell lines, there was reduced overall acetylation of histones in K6/ODC keratinocytes as compared with control keratinocytes. This might be attributed to intrinsic differences between normal primary keratinocytes, which are programmed to terminally differentiate, and immortalized epidermal cell lines, which have undergone genetic changes that circumvent their ability to undergo differentiation, thus permitting continuous proliferation.

Elevated levels of ODC and polyamines at least partially block terminal differentiation in primary keratinocytes *(38)*. However, culturing under conditions normally sufficient to induce differentiation (24 h in 0.12 m*M* calcium medium) had no effect on the reduced level of acetylation mediated by elevated levels of ODC. Moreover, DFMO treatment restored both the normal level of acetylated histones and the induction of the differentiation marker keratin 1 in K6/ODC transgenic keratinocytes triggered to differentiate by the addition of calcium. Our labeling experiments were done in the presence of sodium butyrate, effectively inhibiting HDAC enzymes, implying that polyamines must directly or indirectly inhibit the enzymatic activity of HAT enzymes in normal diploid keratinocytes.

The extent of histone acetylation is the net result of the action of multiple enzymes with acetyltransferase and deacetylase activity. Therefore, we assayed the HAT activity present in homogenates of skin from K6/ODC transgenic mice and their normal littermates, as well as spontaneous skin tumors from ODC/Ras double transgenic mice. Interestingly, increased HAT activity was observed in both the epidermis and dermis, as well as in intact skin of both K6/ODC and ODC/Ras mice *(34)*. Significant, dramatically higher levels of HAT activity were measured in spontaneous tumors from ODC/Ras bigenic mice as compared with normal and K6/ODC skin, as well as non-tumor-bearing

skin from the same ODC/Ras mice. Furthermore, as a result of DFMO treatment, HAT activity in ODC/Ras skin was reduced, and the aberrantly high HAT activity in the tumors was reduced to a level comparable to that detected in nontreated skin. Thus elevated HAT activity in both the skin and tumors of ODC transgenic mice is dependent on polyamine levels.

Similar to the situation for HAT enzymatic activity, HDAC activity was generally found to be increased in the skin of K6/ODC mice relative to normal littermate controls *(34)*. Although there was no obvious change in the level of the predominant HDAC-1 protein in ODC transgenic mouse skin, the possibility that an altered level of one or more of the many other known HDAC enzymes is responsible for the elevated deacetylase activity in ODC skin cannot be ruled out. Interestingly, in contrast to HAT activity, HDAC activity in ODC/Ras tumors was typically lower than that of adjacent non-tumor-bearing skin. Treatment of ODC transgenic mice with DFMO led to decreased intrinsic HDAC activity in non-tumor-bearing skin. Thus, like HAT activity, HDAC activity is also susceptible to regulation by polyamines. However, HDAC activity in tumors was not found to be responsive to DFMO treatment.

The transcriptional coactivator proteins, CBP and p300, play a pivotal role in facilitating synergistic crosstalk between different signaling pathways. They interact with a multitude of nuclear proteins, bridging transcription factors with the basal transcription machinery *(39)*. Moreover, they possess (and recruit proteins with) intrinsic HAT activity *(39)*, altering the local chromatin structure to ultimately influence transcription at individual promoters. It is conceivable that polyamine-mediated modulation of CBP or p300 function could account for many of the diverse and broad effects that polyamines exert on cell growth and differentiation. Indeed, we found that there is increased HAT activity associated with immunoprecipitated CBP/p300 in extracts of K6/ODC transgenic mouse skin relative to normal littermate skin *(34)*. Interestingly, there is no apparent increase in CBP/p300-associated HAT activity in tumors compared with K6/ODC skin or non-tumor-bearing ODC/Ras skin. Furthermore, DFMO treatment has little effect on the CBP/p300-associated HAT activity in the tumors. Thus the high level of HAT activity found in tumors from ODC/Ras transgenic mice does not appear to be primarily contributed by CBP/p300 (or CBP/p300-associated PCAF), but by other HAT enzymes.

As a first step in characterizing the enzyme(s) responsible for the high level of HAT activity in ODC/Ras tumor lysates, in vitro HAT assays were done revealing a striking specificity of lysine acetylation. Using a panel of peptides preacetylated at various lysine residues, it was determined that Lys-12 is a preferred site of acetylation in histone H4 by the HAT activity present in the tumor extracts *(34)*. In this regard, it is interesting that Lys-12 is consistently underused in monoacetylated histone H4 in several mammalian cell types, yet is frequently used in the more highly acetylated H4 isoforms typically associated with actively transcribed DNA *(40)*. Thus the increased HAT activity in ODC transgenic mouse skin and tumors may be associated with hyperacetylation of histones and enhanced transcription at localized regions of the genome. Alternatively, the distinct preference for Lys-12 exhibited by the HAT activity in ODC transgenic tissue may reflect localized remodeling of chromatin into a more heterochromatin-like

structure, perhaps mediated by HAT1. Nuclear HAT1 enzyme has been found to be required for telomeric silencing, mediated solely through Lys-12 acetylation *(41)*. It is thought that acetylation of Lys-12 in histone H4 facilitates binding of silencing proteins that propagate formation of a specialized transcriptionally repressive chromatin structure *(41,42)*. Thus the distinct preference for Lys-12 that is characteristic of ODC transgenic skin and tumors may be indicative of remodeling of localized regions of chromatin into a conformation that is prohibitive to gene transcription.

It is not likely a coincidence that polyamine-mediated enhancement of HAT function occurs in both perpetually growing immortalized cells *(33)* and in the highly proliferative cells in ODC/Ras tumors *(34)*. In contrast to immortalized cells, normal keratinocytes undergo terminal differentiation after only a few cell divisions. This intrinsic difference in cellular programming probably underlies the opposite effects on histone acetylation observed in radiolabeled primary K6/ODC keratinocytes *(34)* as compared with cell lines overexpressing ODC *(33)*. In any case, these varied observations indicate that polyamines can both promote and inhibit the acetylation of histones in epidermal cells, presumably via different molecular mechanisms. This fact, coupled with the demonstration that polyamines modulate both HAT and HDAC enzymes, support the notion that polyamines exert influence on multiple mechanisms controlling histone acetylation (Fig. 1). Neither purified p300 nor PCAF, representatives of two of several distinct classes of nuclear HAT enzymes, were found to be directly stimulated by putrescine or spermidine in cell-free assays *(34)*. Therefore, if the situation with p300 and PCAF is reflective of other HAT enzymes in general, then polyamines likely modulate intrinsic HAT catalytic activity by indirect mechanisms. For instance, polyamines might indirectly regulate enzymatic activity by influencing signaling pathways responsible for the posttranslational modification of HAT or HDAC enzymes. Notably, ODC overexpression leads to greater overall protein phosphorylation in epidermal cells *(43)*, suggesting a major impact on signaling pathways. In this regard, it is intriguing to note that the enzymatic activity and nuclear translocation of casein kinase 2, that includes transcription factors among its substrates, has been found to be stimulated by increased polyamine levels in epidermal cells *(43)*. In addition to potentially affecting posttranslational modification of HAT/HDAC enzymes, polyamines might positively or negatively interfere with interactions between the various components of HAT or HDAC protein complexes. In addition, by modulating subcellular localization or rates of synthesis or turnover of HAT and HDAC proteins, polyamines could effectively manipulate the delicate balance between these enzymes.

Presumably, the abnormally high HAT enzymatic activity characteristic of ODC transgenic mouse skin and tumors is able to target histones in vivo, facilitating enhanced gene expression. However, just as histone deacetylase inhibitors do not promote global changes in gene transcription *(44,45)*, the positive and negative regulation of nucleosomal acetylation exerted by polyamines might be expected to have functional consequences only at localized gene promoters, affecting only a subset of genes. Given that HAT enzymes acetylate non-histone proteins including transactivating factors and components of the basal transcriptional machinery *(37,39)*, aberrantly high HAT activity resulting from ODC overexpression may exert additional major influence

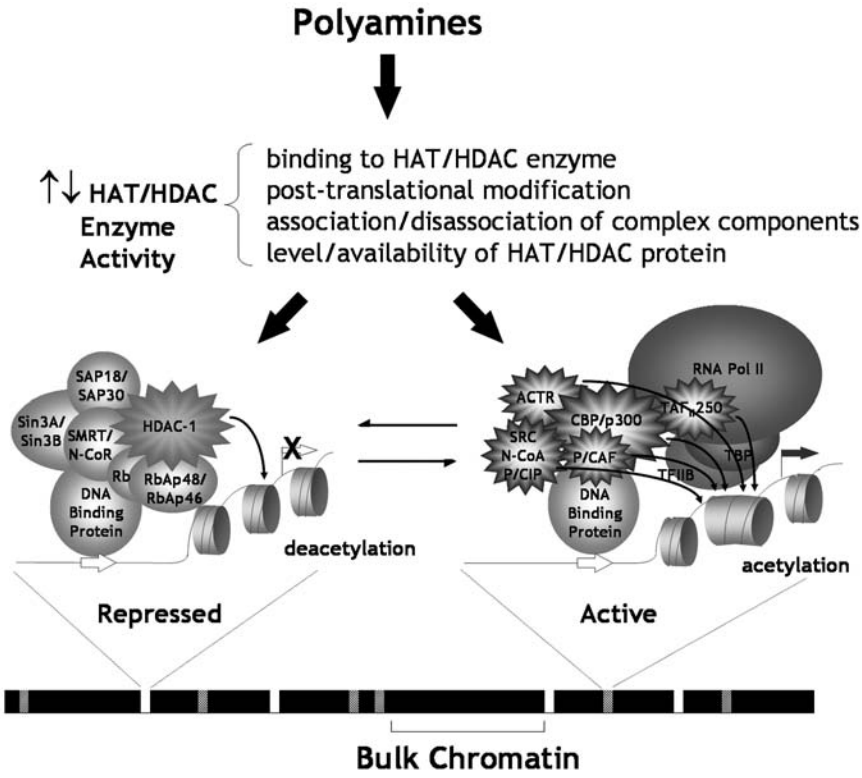

Fig. 1. Potential modes by which polyamines may modulate chromatin remodeling. Transcriptionally competent DNA (vertical bars) comprises a very small amount of the total eukaryotic genome. Whether a gene is transcriptionally silent (white bars) or activated (gray bars) depends largely on the composition of multicomponent histone acetyltransferase/histone deacetylase (HAT/HDAC) protein complexes bound to its regulatory region. Polyamines may influence the function of these protein complexes through direct interaction with HAT or HDAC enzymes. Alternatively, they may indirectly affect enzymatic function by modulating posttranslational modifications, interaction, or the availability of components of the HAT/HDAC complexes. Thus, whether polyamines exert a positive or negative influence on histone acetylation at a localized promoter is likely to be dependent on the specific composition of the regulatory protein complexes governing expression of the gene.

on transcription-related processes. Indeed, we have preliminary evidence suggesting that elevated levels of polyamines affect the acetylation status of multiple non-histone proteins (data not shown). One example is the tumor suppressor protein, p53-overexpression of ODC in skin tissue leads to higher levels of acetylated p53 with concomitant increased binding of p53 to promoters, and associated increases in transcription, of p53 target genes (manuscript in preparation). The future challenge will be to dissect the various regulatory mechanisms modulated by changes in intracellular concentrations of polyamines within the context of individually affected genes. Identification of specific

HAT and HDAC enzymes and gene promoters that are targets of polyamine modulation will provide greater insight into the complex manner by which polyamines regulate histone acetylation and gene expression, and promote tumor development.

It is logical to speculate that the high intrinsic HAT and HDAC activities detected in ODC-overexpressing skin might influence the overall level of acetylated histones in the tissue chromatin. Western analyses using antibodies specific for acetylated histones revealed some modest differences in the acetylation of histones isolated from K6/ODC transgenic skin as compared with normal littermate skin *(34)*. Although noticeable differences in acetylation were not always detected, whenever observed, the differences were consistent between independent preparations of isolated histones. In those cases, fewer acetylated histones H3 and H4 were detected in K6/ODC epidermis, whereas acetylation of histones H3 and H4 was increased in K6/ODC dermis and total skin as compared with histones in normal skin. Moreover, changes in the extent of acetylation of Lys-12 in histone H4 paralleled those observed for hyperacetylated histone H4. These modest changes in overall acetylation suggest that elevated intracellular levels of polyamines do not have a major effect on the acetylation of nucleosomal histones in bulk chromatin. That ODC overexpression induces effects on a small proportion of total histones is consistent with the notion that polyamines promote only very localized changes in the acetylation status of histones in chromatin, perhaps potentiating altered transcription of a small fraction of genes. Moreover, transcriptionally active chromatin actually comprises a very small proportion of the total genome. Thus, any localized effects of polyamines on the acetylation of histones in nucleosomes bound to gene promoters may not be detected within the context of bulk chromatin. This problem is compounded by the fact that recruitment of histone-modifying activities resulting in perturbation of chromatin structure is selectively targeted to specific regions of DNA that may span only one or two nucleosomes *(17)*. Ultimately, the effect of polyamines on histone acetylation will be determined by the composition of the specific regulatory complexes that are recruited *(2)*, as well as the subnuclear compartment in which the gene resides *(46,47)*. Because polyamines apparently influence histone acetylation by multiple mechanisms, changes in cellular polyamine levels can potentially mediate decreased acetylation of nucleosomal histones at one gene promoter, while stimulating increased acetylation at another promoter, resulting in different transcriptional outcomes for those genes (Fig. 1). The ramification for tumor development is that increased polyamine biosynthesis might lead to transcriptional repression of one or more genes controlling cellular proliferation, while simultaneously activating transcription of genes involved in sustaining tumor growth and invasiveness.

Other transgenic mouse models have been developed that feature perturbations of polyamine levels. These include a mouse in which expression of the enzyme spermidine/spermine N^1-acetyltransferase (SSAT) is under control of the natural SSAT promoter *(48)* and another in which the keratin 6 promoter is used to target SSAT overexpression to hair follicles *(49)*. SSAT regulates the catabolism and export of intracellular polyamines. Activation of polyamine catabolism by overexpression of SSAT leads to the production of extremely high levels of acetylated spermidine in both of these models, whereas acetylated spermine remains undetectable. Huge amounts of

putrescine also accumulate in relevant tissues in these mice, presumably because of the back conversion of spermidine as a consequence of SSAT overexpression, combined with the forward conversion of ornithine resulting from a compensatory increase in ODC enzyme activity. Because of this compensatory activation of polyamine biosynthesis, the pools of spermidine and spermine remain relatively unaffected in these SSAT mice, despite the production of large quantities of acetylated spermidine. Significantly, in SSAT mouse prostate, the heightened metabolic flux through the polyamine pathway leads to depletion of pools of acetyl-CoA, which is not only a cofactor for SSAT, but is also critically required for both fatty acid metabolism and the acetylation of histones and other nonhistone proteins. As such, SSAT transgenic mice might be useful for investigating the effects of competition for rate-limiting quantities of acetyl-CoA between enzymes that acetylate proteins and those that acetylate polyamines. Furthermore, given the similarities and differences between ODC and SSAT transgenic mice, comparison of the effect of their respective polyamine pathways on HAT and HDAC enzyme function in tissue might help elucidate the contribution of individual polyamines and their acetylated isoforms to regulating histone acetylation.

Early studies using crude tissue extracts or several "purified" acetyltransferases have reported overlapping specificities of these enzymes for histone and polyamine substrates *(50,51)*. However, in addition to SSAT, the subsequent identification of several different classes of nuclear HAT enzymes, each comprising multiple distinct family members, calls the interpretation of these early studies into question. Notably, the existence of overlapping specificity could conceivably have implications for proliferative diseases that feature revved up production of polyamines, should the excess polyamines successfully compete with protein substrates for rate-limiting HAT enzyme. Likewise, the altered expression of HAT enzymes often observed in tumors *(36,37)* could potentially disrupt the normal balance between nonacetylated and acetylated polyamines. The cloning and expression of individual acetyltransferases should now permit the unequivocal determination of substrate specificities of the various acetylating enzymes.

3. Future Challenges

The evidence points to there being multiple means by which polyamines can both positively and negatively influence the activity of histone acetylating and deacetylating enzymes. Such influence appears to lead to changes in the acetylation status of both histone and non-histone proteins. Fluctuating intracellular concentrations of polyamines do not result in global effects on histone acetylation and gene expression. Therefore, elucidation of the various regulatory mechanisms by which polyamines exert their influence on chromatin-modifying enzymes to modulate gene expression will probably best be accomplished by focusing within the context of individual polyamine-regulated genes. Moreover, it is now possible to individually evaluate the many known HAT and HDAC enzymes for their susceptibility to polyamines and to elucidate the direct or indirect action of polyamines on their function. It will be interesting to determine just how discriminating the polyamines are for the different classes of both HAT and HDAC enzymes. Use of modern molecular biology techniques facilitating the overexpression and isolation of the different protein acetylating enzymes should clarify whether overlapping specificities

exist for histone and polyamine substrates; if so, the relevance for disease should be evaluated. Similarly, models featuring the overexpression of SSAT might be valuable for investigating the effects of competition between enzymes that acetylate proteins and those that acetylate polyamines for rate-limiting quantities of acetyl-CoA.

It has been postulated that normal histone acetylation, probably in conjunction with histone and DNA methylation, serves to imprint the appropriate chromosomal functional status on successive cell generations, thereby contributing to long-term regulation of gene expression *(3,22,23)*. Progeny cells carry an imprint of the histone acetylation pattern present on parental chromosomes before cell division. In this way, acetylation status may act as a marker by which the state of gene activity is carried through from one cell generation to the next. Indeed, histone hyperacetylation has been implicated in the propagation of chromosomal structures during cell division over 80–100 generations *(52)*. Thus, our finding that ODC overexpression can cause altered patterns of histone acetylation may have intriguing implications for ODC-promoted neoplastic growth. It is possible that heightened intracellular levels of polyamines could transiently cooperate with genetic defects to malignantly transform a cell, and then function to sustain the accompanying changes in histone acetylation that may serve to imprint the tumorigenic phenotype such that it is passed along through succeeding cell generations.

Experiments using ODC-overexpressing cell lines and transgenic mice have demonstrated for the first time that polyamines modulate enzymes that manage chromatin structure in mammalian cells and tissue. To date, relatively few chemical classes of inhibitors of histone deacetylases (i.e., sodium butyrate, Trichostatin A, Trapoxin, suberoylanilide hydroxamic acid), and even fewer nonprotein organic modulators of histone acetyltransferases have been identified. Thus the finding that the naturally occurring polyamines can function to modulate HAT and HDAC enzymes is quite significant. Moreover, taken together, the results of studies reviewed here also define a specific mechanism by which aberrant polyamine biosynthesis may lead to inappropriate expression of genes involved in tumorigenesis. As such, this may have important implications for drug development. There is activity for developing inhibitors of histone deacetylases with the intent of promoting histone acetylation and inducing cell-cycle arrest or differentiation in transformed cells. Toward that same end, it is intriguing to consider the possibility of designing compounds that mimic the effect of polyamines in modulating HAT enzymes. On the other hand, these studies also raise a cautionary note with respect to the potential negative ramifications of inappropriate histone acetylation induced by drug treatment. Elucidation of the various ways in which polyamines influence chromatin-modifying enzymes and chromatin structure may provide some insight into the mechanism of action of some anticancer drugs in clinical trials, including some polyamine analogs. Thus, a better understanding of polyamine-mediated modulation of transcription-associated chromatin remodeling might greatly benefit future drug development efforts.

References

1. Wolffe, A. (1998) *Chromatin Structure and Function.* Academic Press, San Diego, CA.
2. Spiegelman, B. M. and Heinrich, R. (2004) Biological control through regulated transcriptional coactivators. *Cell* **119,** 157–167.

3. Hake, S. B., Xiao, A., and Allis, C. D. (2004) Linking the epigenetic 'language' of covalent histone modifications to cancer. *Br. J. Cancer* **90,** 761–769.
4. Fischle, W., Wang, Y., and Allis, C. D. (2003) Histone and chromatin cross-talk. *Curr. Opin. Cell Biol.* **15,** 172–183.
5. Casti, A., Guarnieri, C., Dall'Asta, R., and Clô, C. (1977) Effect of spermine on acetylation of histones in rabbit heart. *J. Mol. Cell. Cardiol.* **9,** 63–71.
6. Bryans, M., Harley, E., and Gilmour, S. K. (1996) Elevated cellular polyamine levels enhance promoter activity in vivo. *Biochem. Biophys. Res. Commun.* **226,** 618–625.
7. Celano, P., Baylin, S. B., and Casero, R. A. (1989) Polyamines differentially modulate the transcription of growth-associated genes in human colon carcinoma cells. *J. Biol. Chem.* **264,** 8922–8927.
8. Wang, J. Y. J., McCormack, S. A., Viar, M. J., et al.(1993) Decreased expression of protooncogenes c-fos, c-myc, and c-jun following polyamine depletion in IEC-6 cells. *Am. J. Physiol.* **265,** G331–G338.
9. Veress, I., Haghighi, S., OPulkka, A., and Pajunen, A. (2000) Changes in gene expression in response to polyamine depletion indicates selective stabilization of mRNAs. *Biochem. J.* **346,** 185–191.
10. Feuerstein, B. G., Pattabiraman, N., and Marton, L. J. (1990) Molecular mechanics of the interactions of spermine with DNA: DNA bending as a result of ligand binding. *Nucleic Acids Res.* **18,** 1271–1282.
11. Matthews, H. R. (1993) Polyamines, chromatin structure and transcription. *BioEssays* **15,** 561–566.
12. Colson, P. and Houssier, C. (1989) Polyamine addition to preparation media induces chromatin condensation, irreversibly at low ionic strength. *FEBS Lett.* **257,** 141–144.
13. Pohjanpelto, P. and Knuutila, S. (1982) Polyamine deprivation causes major chromosome aberrations in a polyamine-dependent Chinese hamster ovary cell line. *Exp. Cell Res.* **141,** 333–339.
14. Snyder, R. D. (1989) Polyamine depletion is associated with altered chromatin structure in HeLa cells. *Biochem. J.* **260,** 697–704.
15. Sunkara, P. S., Chang, C. C., and Prakash, N. J. (1983) Role of polyamines during chromosome condensation of mammalian cells. *Cell. Biol. Int. Rep.* **7,** 455–465.
16. Laitinen, J., Stenius, K., Eloranta, T. O., and Holtta, E. (1998) Polyamines may regulate S-phase progression but not the dynamic changes of chromatin during the cell cycle. *J. Cell. Biochem.* **68,** 200–212.
17. Kadosh, D. and Struhl, K. (1998) Targeted recruitment of the Sin3-Rpd3 histone deacetylase complex generates a highly localized domain of repressed chromatin in vivo. *Mol. Cell. Biol.* **18,** 5121–5127.
18. Pollard, K. J., Samuels, M. L., Crowley, K. A., Hansen, J. C., and Peterson, C. L. (1999) Functional interaction between GCN5 and polyamines: a new role for core histone acetylation. *EMBO J.* **18,** 5622–5633.
19. Megosh, L., Gilmour, S. K., Rosson, D., et al. (1995) Increased frequency of spontaneous skin tumors in transgenic mice which overexpress ornithine decarboxylase. *Cancer Res.* **55,** 4205–4209.
20. Smith, M. K., Trempus, C. S., and Gilmour, S. K. (1998) Co-operation between follicular ornithine decarboxylase and v-Ha-ras induces spontaneous papillomas and malignant conversion in transgenic skin. *Carcinogenesis* **19,** 1409–1415.
21. Hobbs, C. A., Paul, B. A., and Gilmour, S. K. (2003) Elevated levels of polyamines alter chromatin in murine skin and tumors without global changes in nucleosome acetylation. *Exp. Cell Res.* **290,** 427–436.

22. Turner, B. M. (2000) Histone acetylation and an epigenetic code. *Bioessays* **22,** 836–845.
23. Jeppesen, P. (1997) Histone acetylation: a possible mechanism for the inheritance of cell memory at mitosis. *Bioessays* **19,** 67–74.
24. Maison, C. and Almouzni, G. (2004) HP1 and the dynamics of heterochromatin maintenance. *Nat. Rev. Mol. Cell Biol.* **5,** 296–304.
25. Nielsen, S. J., Schneider, R., Bauer, U. M., et al. (2001) Rb targets histone H3 methylation and HP1 to promoters. *Nature* **412,** 561–565.
26. O'Brien, T. G. (1976) The induction of ornithine decarboxylase as an early, possibly obligatory event in mouse skin carcinogenesis. *Cancer Res.* **36,** 2644–2653.
27. Lee, D. Y., Hayes, J. J., Pruss, D., and Wolffe, A. P. (1993) A positive role for histone acetylation in transcription factor access to nucleosomal DNA. *Cell* **72,** 73–84.
28. Dod, B., Kervabon, A., and Parello, J. (1982) Effect of cations on the acetylation of chromatin in vitro. *Eur. J. Biochem.* **121,** 401–405.
29. Estepa, I. and Pestana, A. (1981) Activation by polyamines of the acetylation of endogenous histones in isolated chromatin and nuclei from Artemia. *Eur. J. Biochem.* **119,** 431–436.
30. Libby, P. R. and Bertram, J. S. (1980) Biphasic effect of polyamines on chromatin-bound histone deacetylase. *Arch. Biochem. Biophys.* **201,** 359–361.
31. Vu, Q. A., Zhang, D. E., Chroneos, Z. C., and Nelson, D. A. (1987) Polyamines inhibit the yeast histone deacetylase. *FEBS Lett.* **220,** 79–83.
32. Vergani, L., Mascetti, G., and Nicolini, C. (1998) Effects of polyamines on higher-order folding of in situ chromatin. *Mol. Biol. Rep.* **25,** 237–244.
33. Hobbs, C. A. and Gilmour, S. K. (2000) High levels of intracellular polyamines promote histone acetyltransferase activity resulting in chromatin hyperacetylation. *J. Cell. Biochem.* **77,** 345–360.
34. Hobbs, C. A., Paul, B. A., and Gilmour, S. K. (2002) Deregulation of polyamine biosynthesis alters intrinsic histone acetyltransferase and deacetylase activities in murine skin and tumors. *Cancer Res.* **62,** 67–74.
35. Moshier, J. A., Malecka-Panas, E., Geng. H., Dosescu, J., Skunca, M., and Majumdar, A. P. (1995) Ornithine decarboxylase transformation of NIH/3T3 cells is mediated by altered epidermal growth factor receptor activity. *Cancer Res.* **55,** 5358–5365.
36. Cho, K. S., Elizondo, L. I., and Boerkoel, C. F. (2004) Advances in chromatin remodeling and human disease. *Curr. Opin. Genet. Dev.* **14,** 308–315.
37. Yang, X. J. (2004) The diverse superfamily of lysine acetyltransferases and their roles in leukemia and other diseases. *Nucleic Acids Res.* **32,** 959–976.
38. Lan, L., Hayes, C. S., Laury-Kleintop, L., and Gilmour, S. (2005) Suprabasal induction of ornithine decarboxylase in adult mouse skin is sufficient to activate keratinocytes. *J. Invest. Dermatol.* **124,** 602–614.
39. Kalkhoven, E. (2004) CBP and p300: HATs for different occasions. *Biochem. Pharmacol.* **68,** 1145–1155.
40. Turner, B. M. (1989) Acetylation and deacetylation of histone H4 continue through metaphase with depletion of more-acetylated isoforms and altered site usage. *Exp. Cell Res.* **182,** 206–214.
41. Kelly, T. J., Qin, S., Gottschling, D. E., and Parthun, M. R. (2000) Type B histone acetyltransferase Hat 1 participates in telomeric silencing. *Mol. Cell. Biol.* **20,** 7051–7058.
42. Grunstein, M. (1998) Yeast heterochromatin: regulation of its assembly and inheritance by histones. *Cell* **93,** 325–328.
43. Shore, L. J., Peralta Soler, A., and Gilmour, S. K. (1997) Ornithine decarboxylase expression leads to translocation and activation of protein kinase CK2 in vivo. *J. Biol. Chem.* **272,** 12,536–12,543.

44. Van Lint, C., Emiliani, S., and Verdin, E. (1996) The expression of a small fraction of cellular genes is changed in response to histone hyperacetylation. *Gene Expr.* **5,** 245–253.
45. Sambucetti, L. C., Fischer, D. D., Zabludoff, S., et al. (1999) Histone deacetylase inhibition selectively alters the activity and expression of cell cycle proteins leading to specific chromatin acetylation and antiproliferative effects. *J. Biol. Chem.* **274,** 34,940–34,947.
46. Francastel, C., Schubeler, D., Martin, D. I., and Groudine, M. (2000) Nuclear compartmentalization and gene activity. *Nat. Rev. Mol. Cell Biol.* **1,** 137–143.
47. Gasser, S. M. (2001) Positions of potential: nuclear organization and gene expression. *Cell* **104,** 639–642.
48. Kee, K., Foster, B. A., Merali, S., et al. (2004) Activated polyamine catabolism depletes acetyl-CoA pools and suppresses prostate tumor growth in TRAMP mice. *J. Biol. Chem.* **279,** 40,076–40,083.
49. Coleman, C. S., Pegg, A. E., Megosh, L. C., Guo, Y., Sawicki, J. A., and O'Brien, T. G. (2002) Targeted expression of spermidine/spermine N1-acetyltransferase increases susceptibility to chemically induced skin carcinogenesis. *Carcinogenesis* **23,** 359–364.
50. Libby, P. R. (1978) Calf liver nuclear N-acetyltransferases. Purification and properties of two enzymes with both spermidine acetyltransferase and histone acetyltransferase activities. *J. Biol. Chem.* **253,** 233–237.
51. Cullis, P. M., Wolfenden, R., Cousens, L. S., and Alberts, B. M. (1982) Inhibition of histone acetylation by N-[2-(S-coenzyme A)acetyl] spermidine amide, a multisubstrate analog. *J. Biol. Chem.* **257,** 12,165–12,169.
52. Ekwall, K., Olsson, T., Turner, B. M., Cranston, G., and Allshire, R. C. (1997) Transient inhibition of histone deacetylation alters the structural and functional imprint at fission yeast centromeres. *Cell* **91,** 1021–1032.

6

Role of Polyamines in Regulation of Sequence-Specific DNA Binding Activity

Sripriya Venkiteswaran, Thresia Thomas, and T. J. Thomas

1. Overview

Polyamines are positively charged under physiological ionic and pH conditions. Therefore, the negatively charged macromolecules in the cell, DNA, RNA, and certain proteins are natural targets for their interaction. Polyamine interaction with DNA was considered to be electrostatic in nature and was theoretically interpreted in terms of the counterion condensation theory. Early studies suggested stabilization of duplex DNA by natural and synthetic polyamines, independent of the chemical structure of the polyamine or the sequence of the DNA. More recent studies have revealed polyamine structural specificity, as well as DNA sequence specificity, in addition to the overriding electrostatic interaction. Studies using photoaffinity probes indicate preferential binding of polyamines to bent adenine tracts and TATA elements, suggesting their involvement in the regulation of gene expression. Molecular modeling and experimental studies also indicate sequence-specific binding to GC-rich major grooves. DNA sequence-specific binding of polyamines might also be important in polyamine-induced facilitation of DNA–protein interactions, observed with several transcription factors. The unique contact sites in the interactions between DNA and polyamines are attested by polyamine structural specificity evident in DNA conformational transitions, DNA nanoparticle formation, triplex DNA stabilization, and DNA·RNA hybrid stability. Both polyamine structural specificity and DNA sequence specificity may find applications in polyamine-based drug design, bionanotechnology, and in understanding the mechanism of gene regulation.

2. Introduction

Polyamines are flexible, positively charged, linear molecules present abundantly in the cellular environment *(1,2)*. The predominant natural polyamines—putrescine [$H_2N(CH_2)_4NH_2$], spermidine [$H_2N(CH_2)_3NH(CH_2)_4NH_2$], and spermine [$H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$]—are involved in cell growth and differentiation. Intracellular polyamine concentration is thought to be in the millimolar range. It is estimated that in

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eukaryotes, spermine and spermidine concentrations may be as high as 5 m*M* in the nucleus *(1,2)*. The pK_a values of putrescine (10.8 and 9.4), spermidine (10.8, 9.8, and 8.4), and spermine (10.9, 10.1, 8.8, and 7.9) indicate that all primary and secondary amino groups are protonated under physiological ionic and pH conditions. Because of this polycationic nature of the natural polyamines, negatively charged molecules such as protein, DNA, and RNA are prime targets of their interaction. The distribution of polyamines among the different negatively charged cellular macromolecules in bovine lymphocytes, rat liver, and *Escherichia coli* has been determined *(3)*. Polyamine distribution was calculated by estimating the binding constant of polyamine to DNA, RNA, phospholipid, and nucleotide triphosphates (Table 1). It was found that in bovine lymphocytes and rat liver, the major polyamines were spermine and spermidine, and a major fraction of these were bound to RNA. Free polyamines were estimated to be between 2 and 15% in bovine lymphocytes and rat liver tissue. Putrescine and spermidine were the only polyamines present in *E. coli*, and a majority of these molecules were also associated with nucleic acids. Accurate estimates of free polyamines in different cell types and their fluctuations during cell growth and signaling processes are not available. Free polyamine levels are likely to have an important regulatory role in the modulation of cellular functions. Figure 1 shows the chemical structures of natural polyamines and their commonly used synthetic analogs.

Polyamines are known to facilitate protein–DNA interactions, although it is not clear whether the role of polyamines is driven by site-specific interactions with DNA or the protein *(4–6)*. This function of polyamines results in the regulation of gene expression, as interactions between transcription factors and their response elements are involved. Polyamine-induced conformational alterations in both the DNA component and in the protein are conceivable. Polyamines are also required for catenation of supercoiled DNA by topoisomerases, ensuring fidelity in translation and activation of several enzymes, including kinases, restriction nucleases, and enzymes that are part of the polyamine biosynthetic pathway *(7–9)*.

Polyamines have been shown to stabilize and protect DNA from heat and alkaline denaturation, degradation by enzymes, shear breakage, radiation, and from intercalation of aromatic dyes *(7–9)*. Polyamine-mediated stabilization of DNA helices has been studied for several years *(8,9)*. Polyamines also have a remarkable stabilizing effect on DNA·RNA hybrids, triplex DNA, and other helical structures, such as loops and stems in rRNA, mRNA, and tRNA *(8–12)*. Polyamines have also been shown to induce DNA condensation, aggregation, and resolubilization *(13)*. Polyamine-mediated condensation of chromatin and subsequent protection from nucleases have also been well documented *(14)*. All of these interactions exemplify polyamine binding to nucleic acids by multiple site-specific interactions, some dominated by interactions with phosphate groups, others dominated by base-specific interactions or by a combination of hydrophobic and electrostatic interactions.

3. Interactions of Polyamines With B-DNA and Transition of B-DNA to A-DNA

Early studies on polyamines were interpreted in terms of their binding to the negatively charged phosphate groups. Thus polyamine-induced duplex stabilization,

Table 1
Polyamine Distribution in Bovine Lymphocytes, Rat Liver, and *Escherichia coli*

	Bovine lymphocyte		Rat liver		*Escherichia coli*	
	pH (7.5, 2 m*M* Mg^{2+}, 150 m*M* K^{+})				(pH 7.5, 10 m*M* Mg^{2+}, 150 m*M* K^{+})	
Polyamine Distribution	Spermidine (m*M*) %	Spermine (m*M*) %	Spermidine (m*M*) %	Spermine (m*M*) %	Putrescine (m*M*) %	Spermidine (m*M*) %
(Total)	1.33 (100)	1.58 (100)	1.15 (100)	0.88 (100)	32.2 (100)	6.88 (100)
Free	0.20 (15.0)	0.08 (5.1)	0.08 (7.0)	0.02 (2.3)	12.5 (38.8)	0.26 (3.8)
DNA	0.17 (12.8)	0.29 (18.3)	0.05 (4.3)	0.05 (5.7)	3.0 (9.3)	0.35 (5.1)
RNA	0.76 (57.2)	1.03 (65.2)	0.90 (78.3)	0.75 (85.2)	15.4 (47.9)	6.17 (89.7)
Phospholipid	0.04 (3.0)	0.04 (2.5)	0.07 (6.1)	0.04 (4.5)	0.46 (1.4)	0.05 (0.7)
ATP	0.16 (12.0)	0.14 (8.9)	0.05 (4.3)	0.02 (2.3)	0.84 (2.6)	0.05 (0.7)

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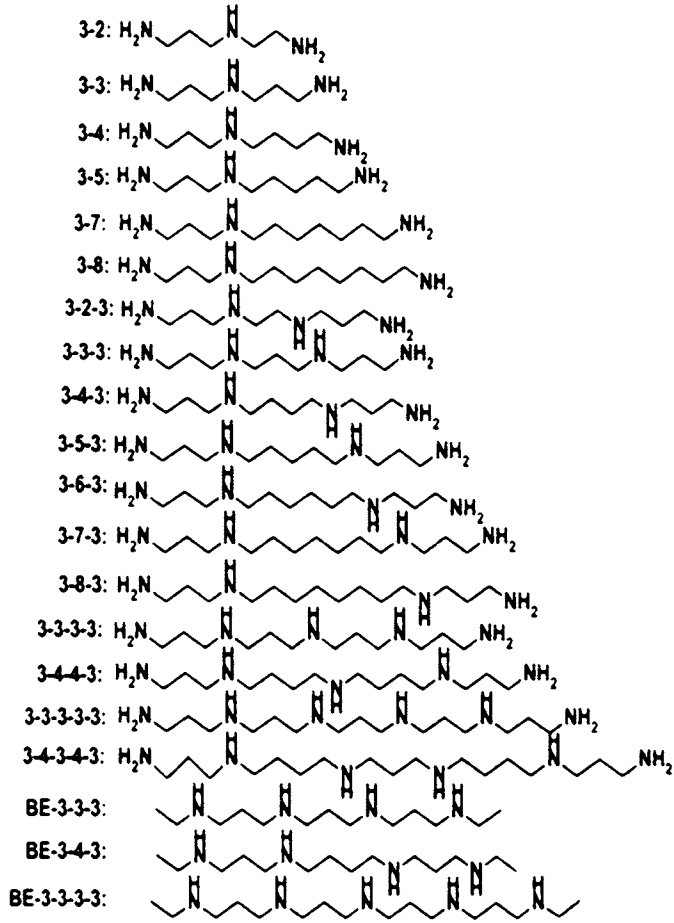

Fig. 1. Chemical structures of polyamines and their analogs.

DNA condensation, and aggregation were thought to be the direct result of interactions of polyamines with DNA phosphate groups involving interstrand and intrastrand binding. However, as it became clear that double-stranded DNA exists in different conformations, depending on the relative water content, nature of counterions, and the nucleotide sequences involved, the differential interaction of polyamines to different forms of DNA became evident *(15)*. The most prevalent form of DNA is the right-handed B-DNA, but the right-handed A-DNA and the left-handed Z-DNA are also observed in X-ray crystallography and solution studies *(16)*. The preferential binding of spermine and spermidine to A-DNA and conversion of B-DNA to the A-form in the presence of these polyamines was reported as early as 1979 *(17)*. Conversion to A-form was detected by circular dichroism spectroscopy and the effects were observed in water–ethanol mixtures. Interestingly, putrescine, cadaverine, and

hexamethylene diamine did not follow the pattern of spermidine and spermine, but stabilized B-DNA.

Molecular modeling studies by Zakrezewska and Pullman *(18)* indicated that spermine binding depended on DNA conformation and base sequence. A model for the interactions of polyamines with B-DNA simulated by theoretical calculations indicated binding to the minor groove of DNA (*18,19*). In molecular models of Suwalsky et al. *(20)* spermine was modeled to bridge two backbone strands across the B-DNA minor groove. However, molecular dynamics modeling by Feuerstein et al. *(21)* found the major groove of alternating purine/pyrimidine sequence to be the most favorable sites for spermine binding and the association of spermine binding with DNA bending. Binding of spermine along the phosphate backbone was the least favorable interaction. Comparison of $d(GC)_5$-$d(GC)_5$ heteropolymer with the homopolymers $(d(G)_{10}$-$d(C)_{10}$ showed continuous interaction with heteropolymer but not homopolymer.

X-ray crystallography of the d(GTGTACAC) octamer duplex showed a unique positioning of spermine in its A-DNA conformation *(22)*. Spermine bound to the floor of the major groove by hydrogen bonding to GTG of one strand, assumed an S shape, and bound to the corresponding bases on the opposite strand. The methylene groups of spermine formed a hydrophobic cluster with the methyl group of thymine and the O6 atoms of the guanine of the TGT sequence. The A-DNA dodecamer, d(CCGGGCCCCGG), crystallized in the presence of spermine in three polymorphic forms, illustrating the different modes of spermine binding *(23)*. These different polymorphic forms represent differences in hydration states, widths of major grooves, and the extent of DNA bending. Multiple spermine molecules shield areas of highest phosphate density, allowing crystal-packing forces to condense, bend, and twist duplex DNA. Binding of spermine with A-DNA was classified as backbone/groove, major groove, minor groove, and backbone-only interactions *(15)*. Two amine/imine functionalities of spermine can interact with bases in the deep groove, whereas the remaining two bind to two phosphate groups of the reference duplex and a neighboring duplex in the crystal structure of the dodecamer.

Figure 2 illustrates electron density maps of three different modes of spermine binding to DNA. Although spermine has been found in several A-DNA crystal structures and in a structure of a DNA·RNA hybrid, spermine has been found only rarely in the B-DNA crystal structure.

Spermine binding to the GTG site of a B-DNA hexamer d(CGGTGG)/d(CCACCG) suggests an opening of the base pair T/A resulting in a novel non-Watson–Crick hydrogen bonding scheme between adenine and thymine in the region *(24)*. Figure 3 shows a model of spermine interaction with the hexameric DNA as derived from the crystal structure. Spermine stabilizes the sheared base pair by “pinching together” the minor groove across the G5 and C10 phosphates. Because GTG base triplet appears five times more frequently in regulatory regions, it is suggested that crystallography in the presence of spermine might have captured a general mechanism for protein–DNA recognition, marked by the opening of a base pair.

Although a number of crystallographic and molecular modeling studies indicate site-specific binding of spermine to G/C rich sequences, studies using photoaffinity probes indicate a preferential binding to A-tracts in double-stranded DNA sequences. Lindemose

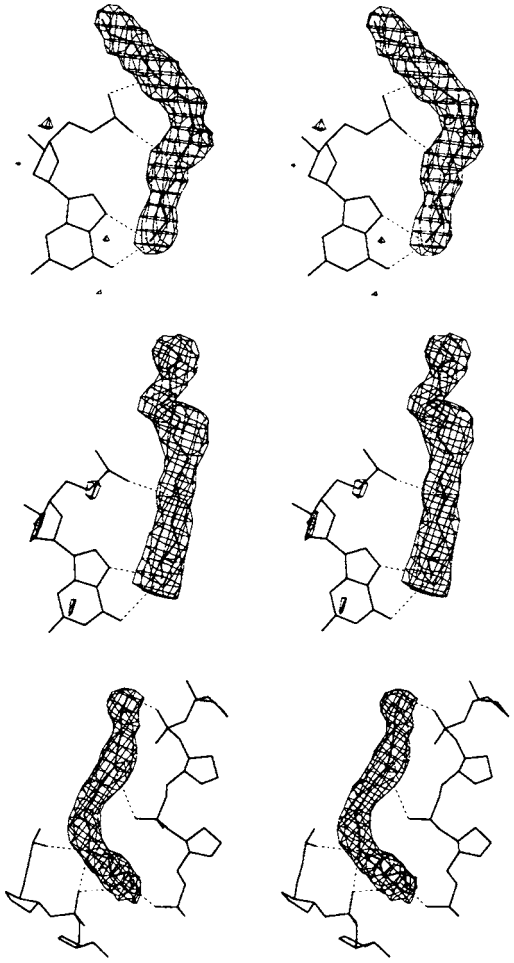

Fig. 2. Electron density maps of spermine molecules found in the crystal lattice of the Ortho 1 (top) and Ortho 2 (middle and bottom) forms of d(CCGGGCCm^{5}CGG). Broken lines indicate possible hydrogen bonding between spermine amino and imino groups with DNA acceptor atoms. In Ortho1, the single spermine is bound to the terminal guanine, interacting with O-6 and N-7 atoms through the central imino atoms N-5 and N-10. In the second orthorhombic form, Ortho2, one spermine molecule is bound at the opposite end of the helix to G10 and interacts through its terminal amino group nitrogen atom, N-1. Interactions of the central imino group nitrogen atom, N-5, with the adjacent phosphate are ambiguous. The second spermine molecule in Ortho 2 interacts with phosphate groups on opposite sides of the major groove. (Reprinted from ref. *23* with permission from Elsevier.)

et al. *(25)* conducted uranyl photocleavage and DNAse I cleavage of *E. coli tyr* T promoter, cytomegalovirus (CMV) promoter, and other double-stranded DNA containing A/T tracts. In these studies, putrescine, spermidine, and spermine showed preferential binding to A/T sequences, although spermine was 100-fold more efficient than putrescine in

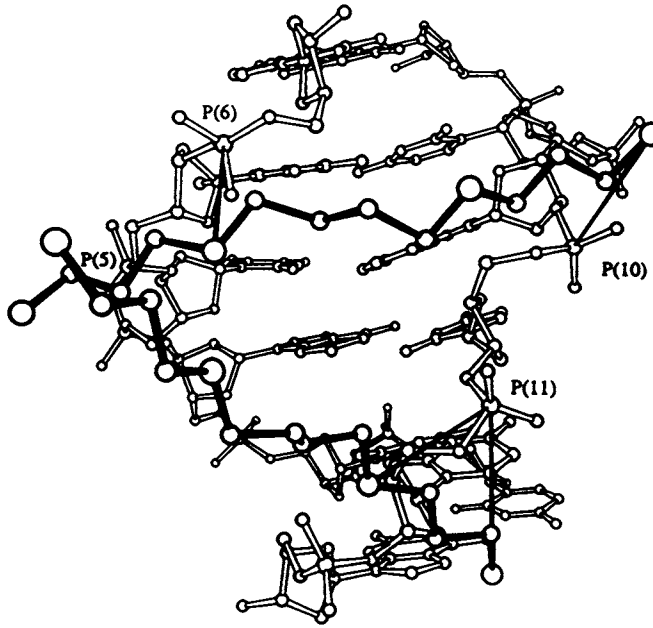

Fig. 3. Symmetry related spermine A molecules (dark bonds) span the minor groove in the vicinity of the α^+ phosphates and stabilize the sheared T/A base pair. Thin lines indicate close contacts (<4′, after subtracting the van der Waals radii of the phosphates and the amino groups) between anionic phosphates and cationic amino groups. (Reprinted from ref. *24* with permission from Oxford University Press.)

protecting the A/T tract DNA from cleavage. Studies using a photoaffinity polyamine (azidonitrobenzoyl)spermine (ANB-spermine) that could be activated by light indicated preferential binding to the TATA sequence *(26)*. Other studies using imino proton exchange rates and ethidium displacement have also indicated preferential binding to A/T sequences *(27–29)*. Preferential binding of polyamines to the TATA sequence is particularly significant because of the importance of the TATA element in the recognition by RNA polymerase in transcription. Binding of polyamines to the TATA element may act as a mechanism to suppress transcription and this repression might be removed when polyamines are acetylated.

4. B-DNA to Z-DNA Transition and Stabilization

Molecular structure of left-handed Z-DNA was established 25 yr ago by single crystal X-ray crystallography of alternating purine/pyrimidine sequences *(16)*. Z-DNA has a zig-zag arrangement of the sugar phosphate backbone with only deep minor grooves and no discernible major groove (Fig. 4). In contrast, the phosphate groups lie on a smooth helical line in B-DNA with minor and major grooves. Initially, Z-DNA was thought to be a nonphysiological structure, because 3 to 4 *M* NaCl was required to induce Z-DNA in solution. However, later studies revealed that μ*M* concentrations of polyamines could convert B-DNA to Z-DNA in alternating purine/pyrimidine

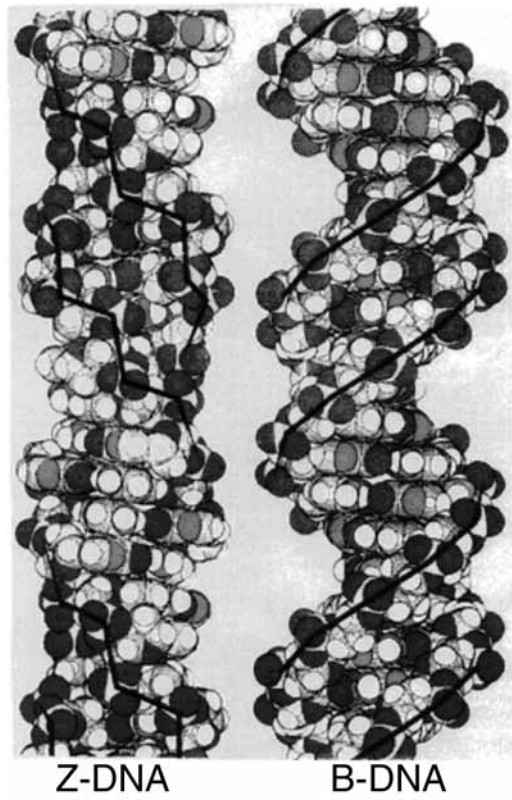

Fig. 4. The "information-rich" residues that allow sequence-specific recognition of the major groove of the B-DNA lie on the convex surface of left-handed Z-DNA helix. The two DNA strands of each duplex are highlighted by a *solid black line.* The "zigzag" nature of the Z-DNA backbone is clearly seen (Reprinted from ref. *16*; permission conveyed through Copyright Clearance Center, Inc.).

sequences *(30)*. The fluctuating concentrations of polyamines and supercoiling of transcriptional domains might work together with DNA sequence-specificity in inducing and propagating Z-DNA. Indeed, the intrinsic affinity of spermine for Z-DNA was found to be 10-fold higher for d(CA/TG) than for d(CG) dinucleotide, and both greater than that of B-DNA *(31)*.

The packing of spermine–DNA complexes in a pure-spermine form of Z-DNA crystals suggested that the molecular basis for the tendency of spermine to stabilize compact DNA structures derived from the capacity of spermine to interact simultaneously with several duplexes. This capacity is maximized by the flexibility of the methylene-bridging regions of spermine. The length and flexibility of spermine and the dispersion of charge–charge, hydrogen-bonding, and hydrophobic-bonding potential throughout the molecule maximize the ability of spermine to interact simultaneously with different DNA molecules. Although major and minor groove binding of spermine to DNA have been demonstrated in various studies, recent modeling studies emphasize the mobility

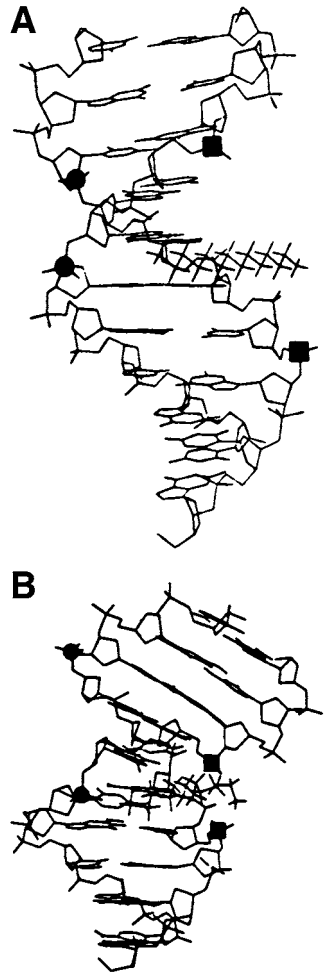

Fig. 5. Views into the major groove of d(G-C)5/d(G-C)5 with spermine in place before **(A)** and after **(B)** energy minimization. Note the decrease in distance across the major groove (■) and the increase across the minor groove (●) after energy minimizations was performed. Squares and circles represent the same points on the helix, and are included for comparison between **(A)** and **(B)**. (Reprinted with permission from ref. *33*.)

of the spermine molecule between major and minor grooves and phosphate backbone binding sites *(32)*. The competition of polyamines with monovalent and divalent cations, the width and other spatial parameters of major and minor grooves, and the functional groups of base sequence are all involved in "fixing" the polyamine binding sites. For example, the conformational energy calculations of Feuerstein et al. *(33)* indicate maximal interactions between proton acceptors on the oligomer and proton donors on spermine (Fig. 5). When spermine was docked into the major groove of

B-DNA, dynamic changes in DNA structural parameters, such as DNA bending over the major groove containing spermine, alterations in oligomer sugar puckering, and interstrand phosphate distances, occurred. Although the sequence used has the capacity to form Z-DNA, the experimental parameters were set for B-DNA. In contrast, structures of Z-DNA in which the crystals are produced in the presence of spermine, shows that the high prevalence of minor grooves and the rigidity of the Z-DNA backbone structure provides opportunity for minor groove binding of the spermine in $d(CGCGCG)_2$ hexamer crystals *(34)*. Thus, crystal structure analysis and molecular modeling studies provide data for the specific contexts revealing the preferential binding of spermine to major or minor grooves of DNA.

Behe and Felsenfeld *(30)* first showed that physiologically compatible concentrations of spermidine and spermine could induce and stabilize the Z-DNA. Thomas and Messner *(35)* further investigated the structural specificity effects of spermidine homologs in the induction and stabilization of the Z-DNA for poly(dG-m^5dC)/poly(dG-m^5dC). They found that spermidine was fourfold more efficient than a homolog with two additional $–CH_2–$ groups in the methylene chain, in inducing and stabilizing the Z-DNA (Fig. 6). In addition to conventional spectroscopic techniques, a monoclonal anti-Z-DNA antibody-based enzyme-linked immunosorbent assay was developed to detect Z-DNA formation. Using the enzyme-linked immunosorbent assay technique, Z-DNA formation was also detected in $(dG\text{-}dC)_n$ sequences inserted in a plasmid DNA *(36)*. In contrast, there was no Z-DNA formation in the control plasmid, thereby showing sequence-specific interaction of polyamines with the $(GC)_n$ insert. Structural specificity effect was also evident in the efficacy of three spermidine homologs to induce the Z-DNA conformation in the plasmid with insert sequences. These results indicate that spermidine and spermine are capable of provoking the left-handed Z-DNA conformation in small blocks of $(dG\text{-}dC)_n$ sequences embedded in a right-handed B-DNA matrix. Because blocks of $(dG\text{-}dC)_n$ sequences are found in genomic DNA, conformational alterations of these regions to the Z-DNA form may have important gene regulatory effects in the presence of polyamines.

5. Sequence-Specific Effects of Polyamines on Protein–DNA Interaction

The consequences of polyamine binding to DNA can be manifested in DNA replication and transcription. In 1980, Geiger and Morris *(37)* demonstrated the requirement of polyamines for DNA synthesis in *E. coli*. They found that DNA synthesis was reduced in polyamine-starved *E. coli*, and that cells could be rescued by the addition of spermidine. This result suggested that polyamines functioned as cofactors in the DNA replication machinery in *E. coli*, and probably facilitated the interaction of trans-factors involved in DNA replication. Porter and Bergeron *(38)* showed structural specificity effects of polyamines in supporting the growth of L1210 leukemia cells treated with α-difluoromethylornithine, a specific inhibitor of putrescine biosynthesis. They found that three of six spermidine homologs supported cell growth during a 48-h incubation in the presence of α-difluoromethylornithine, indicating that a two-carbon extension of the spermidine structure was tolerated for biological function.

Panagiotidis et al. *(39)* studied the effect of polyamines on complex formation of sequence-specific DNA binding proteins with DNA. They found that spermidine

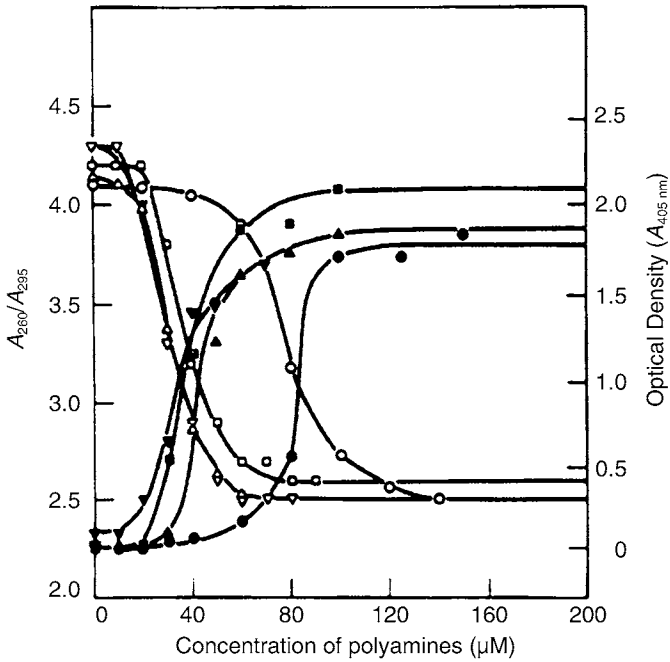

Fig. 6. Immunological and ultraviolet spectroscopic detection of the B-DNA to Z-DNA transition of poly(dG-m^{5}dC)-poly(dG-m^{5}dC) in the presence of polyamines in 50 m*M* NaCl, 1 m*M* Na cacodylate, and 0.15 m*M* EDTA (pH 7.4). The polynucleotide was incubated with different concentrations of polyamines for 30 min and added to the microtiter plates. Enzyme immunoassay was conducted by subsequent addition of monoclonal Z-DNA antibody Z22, alkaline conjugated immunoglobulins, and the enzyme substrate as described by ref. *35*. Optical density was read at 405 nm ($A_{405\ nm}$) with a microplate autoreader. $A_{405\ nm}$ is plotted against the concentration of AP2 (●), AP3 (▲), AP4 (spermidine) (▼), and AP5 (■) added to the polynucleotide. In ultraviolet spectroscopy, the absorbance ratio of poly(dG-m^{5}dC)-poly(dG-m^{5}dC) (A_{260}/A_{295}) was monitored at different concentrations of AP2 (○), AP3 (Δ), AP4 (▽) and AP5 (□). (Reprinted with permission from ref. *35*.)

increased the effectiveness of binding of the ICP-4, a herpes simplex virus gene regulator to its specific binding sequence on the DNA. They also found that the association constants of the transcription factor to its specific site on the DNA were increased in the presence of polyamines. The authors concluded that this increase in complex formation occurred as polyamines increased the affinity of the protein for its cognate DNA site by increasing the initial binding step. A study by Thomas and Kiang *(5)* demonstrated a 22-fold increase in the binding of the estrogen receptor (ER) to poly(dA-dC)/poly(dG-dT) in the presence of spermidine. In the same study, they compared the affinity of the same ER with poly(dA-dT)-poly(dA-dT) and found only a threefold increase in the presence of polyamines, suggesting a sequence-specific effect of polyamines on protein–DNA binding. The authors reasoned that this specific preference

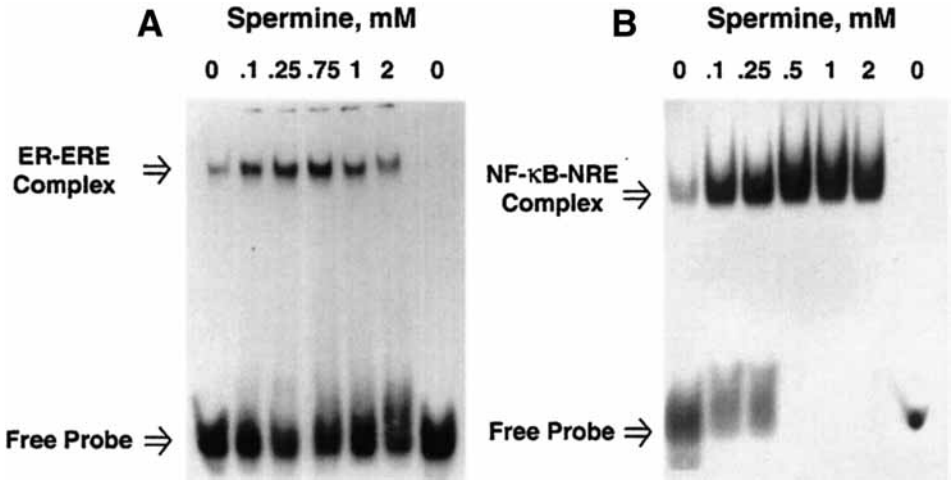

Fig. 7. Effect of spermine on estrogen receptor (ERα) and nuclear factor κB (NF-κB) binding to their response elements. **(A)** Electrophoretic mobility shift assay (EMSA) was conducted using human recombinant ERα and ^{32}P-labeled ERE (estrogen response element) oligonucleotide in the presence of increasing concentrations of spermine. The last lane was loaded with free probe (labeled ERE without extract). The reaction mixture was then loaded on a 6% polyacrylamide gel, electrophoresed and autoradiographed. **(B)** EMSA was conducted using cellular extract from MCF-7 cells and ^{32}P-labelled NRE (nuclear factor response element) in the presence of increasing concentrations of spermine. (Reprinted with permission from ref. *6*.)

of the ER to the polynucleotide might be the result of polyamine-induced conformational change in the structure of the polynucleotide to the Z-DNA form. This is particularly important in the context that potential Z-forming sequences have been detected in enhancer elements of many genes.

The ability of polyamines to modulate the affinity of sequence-specific effects of protein–DNA interaction was also reported using ER, progesterone receptor, and the vitamin D receptor *(2,6,8)*. Shah et al. *(5)* showed the ability of spermine to increase the association of the CBP/p300 coactivator protein with ER and nuclear factor-κB (NF-κB) in MCF-7 breast cancer cells in a concentration-dependent manner. The same authors also found that ER and NF-κB binding to its consensus sequence on DNA increased in the presence of polyamines (Fig. 7). The increased DNA–protein and protein–protein interactions observed because of increased polyamine levels resulted in increased transcription of estrogen responsive reporter genes (Fig. 8). Thus polyamines might be important in the formation of multiprotein complexes on promoter DNA as part of the transcriptional initiation process. A recent study *(25)* using uranyl photocleavage experiments shows that spermine binds specifically to bent A-tracts in DNA and that A-tracts are present near the binding site of NF-κB (Fig. 9). The authors speculate that this binding of the polyamine to the bent A-tracts

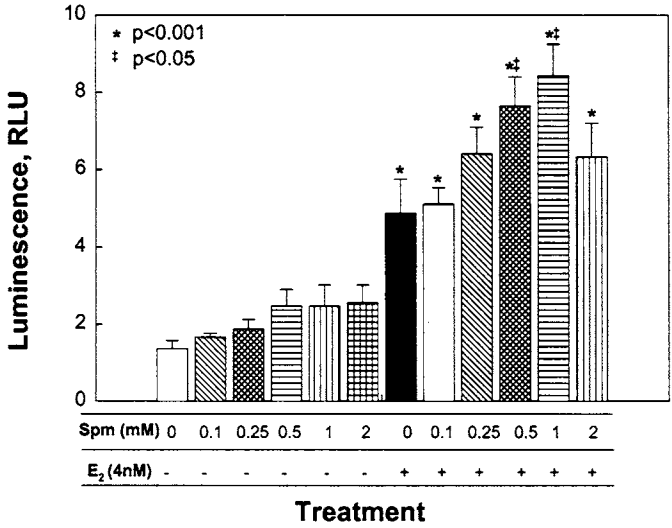

Fig. 8. Effect of spermine on ERα-mediated transcription of a luciferase reporter gene. Plasmids pGL3-4(EREc38) and pRL-TK control vector were co-transfected in MCF-7 cells. After 24 h, cells were treated with increasing concentrations of spermine in the absence or presence of 4 n*M* estradiol. Cells were harvested 6 h after treatment and assayed for luciferase activity. Relative light units were normalized for each sample by dividing the firefly luciferase activity by the renalia luciferase activity. Data represent average ± SE from three separate experiments. Statistical significance was determined by analysis of variance followed by Tukey's test for significance for spermine and estradiol treatment groups compared to combinations. *Statistically significant from control group ($p < 0.001$). ‡Statistically significant compared to estradiol treatment group ($p < 0.05$). RLU, relative light units. (Reprinted with permission from ref. *6*.)

facilitate the "sliding" of the transcription factor to its specific site on DNA from its nonspecific site.

Kuramoto et al. *(40)* reported that DNA binding of nuclear transcription factors with the leucine-zipper motif is modulated by polyamines. Addition of spermidine and spermine increased the DNA-binding activity of the AP-1 transcription factors in whole brain extracts in a concentration-dependent manner. A similar but less potent increase in binding is observed with cyclic adenosine monophosphate response element-binding protein. In contrast, binding of c-MYC protein to a cognate sequence was not altered by polyamines. Lewis et al. *(41)* found that the binding of activating transcription factor-2 to its binding site in cyclin D1 promoter was enhanced by spermine by a mechanism involving both a cell signaling pathway, as well as direct effects on DNA–protein interactions. Very little is known about polyamine-induced changes in protein conformation, although spermine has been reported to change the conformation of a peptide *(42)*. Thus polyamine-induced changes in DNA–protein interactions and conformational transitions are likely to have a range of consequences in DNA–protein recognition and regulation of gene expression.

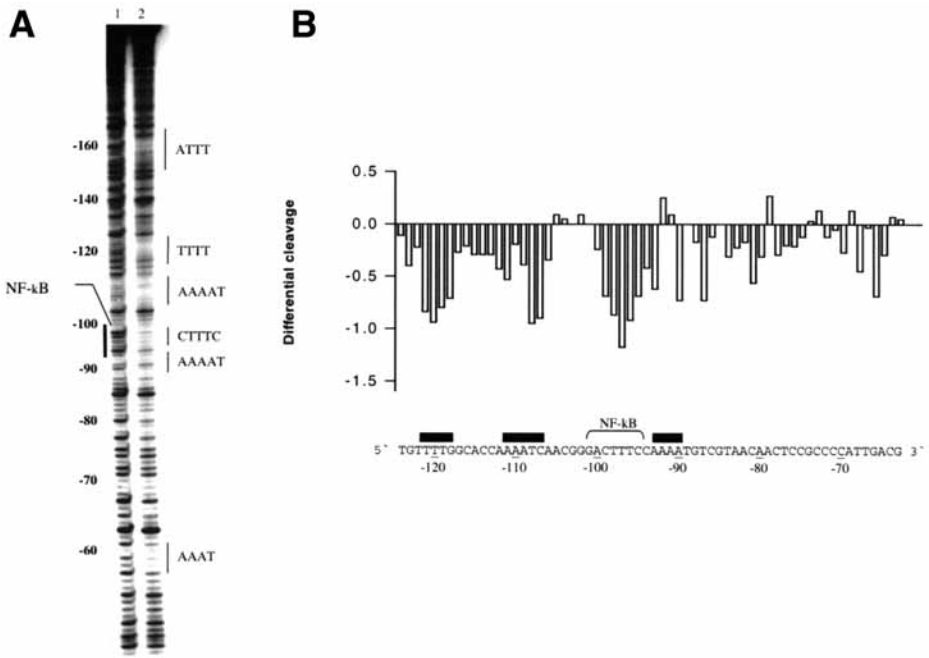

Fig. 9. Effect of spermine on DNAse I cleavage of the human CMV promoter DNA. **(A)** A typical autoradiogram is shown in the absence and in the presence of 8 m*M* spermine (lanes 1 and 2, respectively). The autoradiogram covers from –60 to –160 bp upstream from the transcription start site. Boldface numerals on the left-hand side indicate base positions with respect to transcription start site. The position of AT-rich sequences and NF-κB binding site are also shown. Black boxes indicate the position of A-tracts. **(B)** Differential cleavage plot comparing the susceptibility of the human CMV promoter to DNAse I in the absence and presence of 8 m*M* spermine. The analysis covers from –70 to –120 bp upstream from the transcription start site. Black boxes indicate the position of A-tracts. (Reprinted from ref. *25* with permission from Oxford University Press.)

6. Double Helix Stabilization: DNA·DNA, DNA·RNA, and RNA·RNA

One of the earliest discoveries about polyamine–DNA interactions was the observation that polyamines could stabilize the DNA double helix. In 1962, Tabor *(43)* described the ability of spermine and other polyamines to protect DNA from heat-induced helix $\rightarrow$ coil transition. Subsequently, several studies confirmed that natural and synthetic polyamines were capable of increasing the helix $\rightarrow$ coil transition or melting temperature (T_m) of DNA. Thomas and Bloomfield *(44)* conducted a detailed study of the ionic and structural effects of polyamines on the T_m of calf thymus DNA. Using a series of spermidine homologs of the structure, $NH_3(CH_2)_3NH_2(CH_2)_nNH_3^{3+}$, where "n" varied from 2 to 8, they found modest but significant structural effects of polyamines on stabilizing duplex DNA. (*See* Fig. 1 for chemical structures of some of the polyamine homologs and analogs mentioned in this chapter.) A monovalent ion, Na^+, competed with polyamines in interacting with DNA. Braunlin et al. *(45)* quantified this

ion competition effect of monovalent ions and multivalent polyamines by measuring binding constants using equilibrium dialysis experiments.

Venkiteswaran et al. *(12)* recently studied the effects of natural and synthetic polyamines on the thermal stability of DNA·DNA, DNA·RNA, and RNA·RNA duplexes comprising a therapeutic antisense oligonucleotide targeted toward the initiation codon of the human epidermal growth factor-2 (HER-2) mRNA. The antisense oligonucleotide had the following sequence: 5′-CTCCATGGTGCTCAC-3′. A major goal of this study was to examine how polyamines interacted with DNA·RNA hybrids during the use of antisense oligonucleotides for therapeutic purposes. The *HER-2* gene is overexpressed in several cancers, and breast cancer patients with HER-2 overexpression have poor prognosis. Efficiency of antisense technology (i.e., the ability of a small oligonucleotide to block the translation of targeted genes) depends to a large extent on the stability of DNA·RNA hybrid formed in vivo. In addition, the antisense oligonucleotides have to be stable against nuclease digestion. A commonly used technique to impart stability of the oligonucleotides is to substitute an oxygen atom for a sulfur atom. The resulting phosphorothioate oligonucleotides are stable; however, DNA·RNA hybrids formed with phosphorothioate oligonucleotides have lower thermal stability compared with that formed with the parent phosphodiester oligonucleotides. Therefore, the effects of polyamines were studied on hybrids formed with both phosphodiester and phosphorothioate oligonucleotides.

Polyamines increased the T_m of DNA·DNA, DNA·RNA, and RNA·RNA in a concentration-dependent manner (Fig. 10). As the concentration of spermine increased, the melting curve shifted to the right, indicating an increase in the T_m of the hybrid. With respect to the cationicity of polyamines, we found that putrescine was the least efficient and spermine the most efficient in stabilizing hybrids (Table 2). For example, ΔT_m (T_m in the presence of polyamines $-T_m$ in the absence of polyamines) of the DNA·RNA hybrid was negligible (1°C) in the presence of 1 m*M* putrescine; however, ΔT_m of the same hybrid increased to 10.5°C in the presence of 1 m*M* spermine. Another important finding from this study was that the spermine effect on DNA·RNA hybrid was much larger (ΔT_m = 11°C) than that on DNA·DNA duplex (ΔT_m = 3.5°C). RNA·RNA duplex showed a slightly higher increase in T_m with 1 m*M* spermine (ΔT_m = 14.6°C). These data indicate a preferential stabilization of DNA·RNA and RNA·RNA duplexes compared with the DNA·DNA duplex. These studies demonstrate the differential effects of polyamines in stabilizing nucleic acid helices, depending on the structure of the sugar moiety, whether it is a deoxysugar or ribosugar. However, more research is needed to confirm whether these differences are related to the relative affinity of polyamines to different forms of duplex. As in other studies, substitution of the primary amino group of the polyamines by an ethyl group resulted in a reduction of the hybrid stabilizing potential of the modified compound compared with its unmodified counterpart.

7. Triplex DNA Stabilization

Triplex DNA has assumed much attention during the past three decades because of the potential gene regulatory effects of triple helix-forming oligonucleotides, and subsequently for their use as therapeutic agents *(46)*. Triplex forming poly(purine)/poly

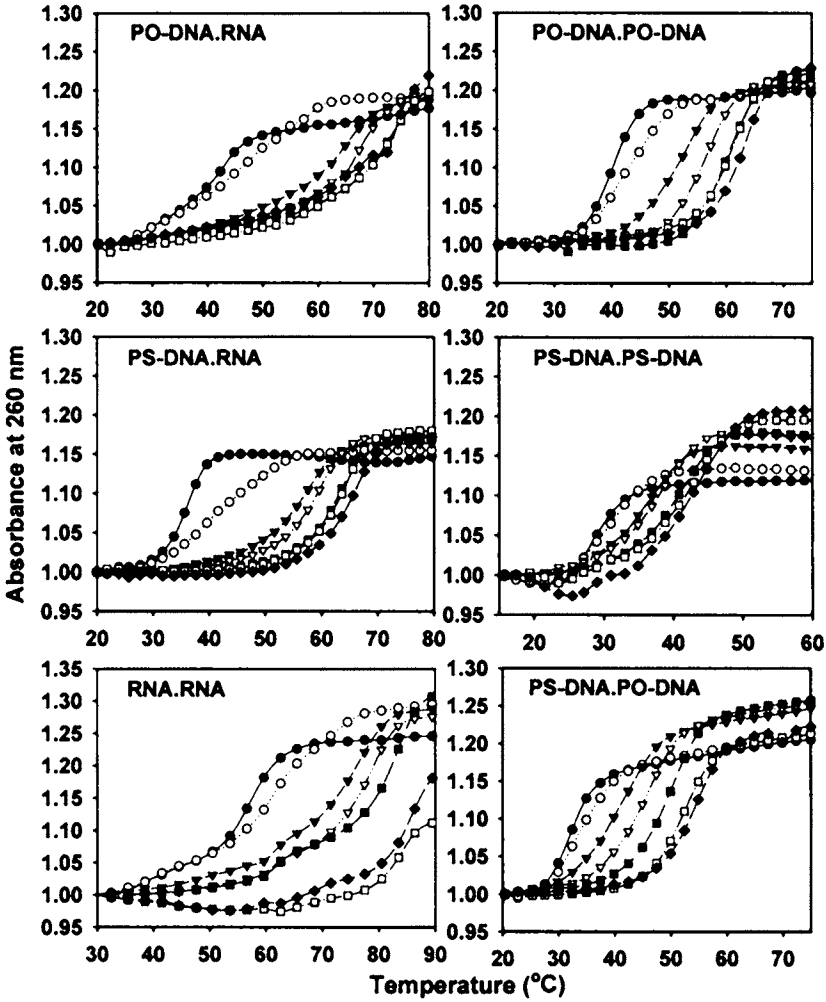

Fig. 10. Melting profile of the PO-DNA·RNA, PS-DNA·RNA, PO-DNA·PO-DNA, PS-DNA·PS-DNA, PS-DNA·PO-DNA, and RNA·RNA helices. The concentrations of spermine used were 0 (●), 1 (O), 5 (▼), 10 (▽), 25 (□), 50 (■), and 100 μ*M* (♦). The melting temperature measurements were carried out in 10 m*M* cacodylate buffer at a heating rate of 0.5°C/min for all the hybrid helices. (Reprinted with permission from ref. *12*. © 2005 American Chemical Society.)

(pyrimidine) sequences are found in the promoter elements of several genes, including *c-myc*, HER-2, and *Ets-2*. A ribbon model representation of triplex DNA is shown in Fig. 11. Triple helix formation involves the binding of a short oligonucleotide via hydrogen bonding to the major groove of a double helix. The widely accepted structural model for polypurine/(polypyrimidine)$_2$ triple helix is based on X-ray fiber diffraction studies. In this structural model, an A-form polypurine/polypyrimidine duplex

Table 2
Effects of Polyamines on the Stability of Various Hybrids in the Presence of 150 m*M* Na^+

	T_m (°C)[a]					
Concentration polyamines (m*M*)	PO-DNA·RNA	PS-DNA·RNA	PS-DNA·PO-DNA	PO-DNA·PO-DNA	PS-DNA·PS-DNA	RNA·RNA
Putrescine						
0	62.0	53.4	52.8	61.4	45.8	72.8
0.5	62.5	54.0	53.3	62.9	45.8	74.4
1	63.0	54.4	53.8	62.9	45.8	75.8
ΔT_m[b]	1.0	1.0	1.0	1.5	0	3.0
Spermidine						
0	61.9	53.4	52.4	60.9	44.9	73.9
0.5	65.0	57.9	53.4	62.0	45.9	78.9
1	67.5	59.5	54.4	63.0	46.9	82.4
ΔT_m	5.6	6.1	2.0	2.1	2.0	8.5
Spermine						
0	61.5	54.2	52.4	61.4	44.9	72.8
0.5	69.9	63.7	54.3	63.5	46.4	85.3
1	72.0	65.2	55.9	64.9	47.4	87.4
ΔT_m	10.5	11	3.5	3.5	2.5	14.6

[a]The T_m measurements were carried out in 150 m*M* Na cacodylate buffer (140 m*M* NaCl added to 10 m*M* Na cacodylate buffer). The reproducibility of the T_m values was ± 1°C for three or four measurements.

[b]$\Delta T_m = T_{m(1\ mM)} - T_{m(0)}$, where $T_{m(1\ mM)}$ is the T_m of the various hybrids in the presence of 1 m*M* polyamine and $T_{m(0)}$ is the T_m of the hybrid in 150 m*M* Na cacodylate buffer, in the absence of polyamine. Reprinted with permission from ref. *12*.

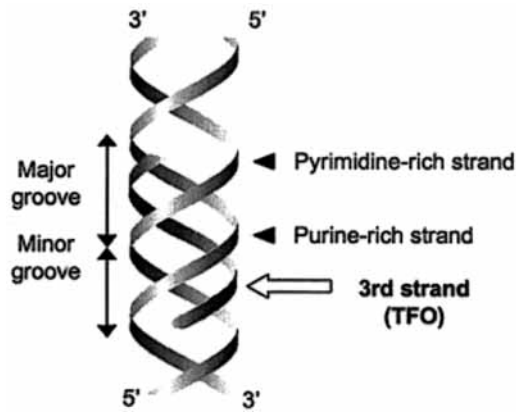

Fig. 11. Representation of a triplex forming oligonucleotide binding in the major groove of DNA. (Reprinted from ref. *46* with permission from Oxford University Press.)

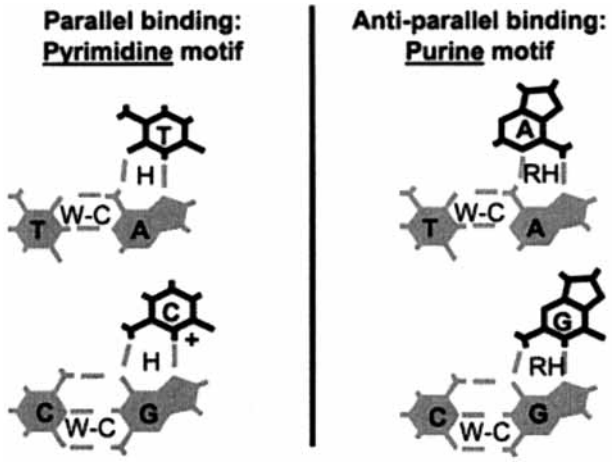

Fig. 12. Recognition motifs for triplex forming oligonucleotide binding to DNA. Note the requirement for protonation of cytosine (at N^3) in the pyrimidine motif. W-C, Watson–Crick hydrogen bonds; H, Hoogsteen hydrogen bonds; RH, reverse Hoogsteen hydrogen bonds. (Reprinted from ref. *46* with permission from Oxford University Press.)

formed by Watson–Crick base pairing binds to a polypyrimidine strand in the major groove. The second polypyrimidine strand binds to the duplex through Hoogsten base pairing between thymine and A:T base pairs and between protonated cytosine and G:C base pairs, giving rise to (T*A:T) and (C^+*G:C) triplets, respectively. However, because the protonation of cytosine in the third strand is an essential requirement, triplex formation involving these triplet sequences can only occur at the acidic pH of 5.6–6.0. The hydrogen bonding scheme for triplex formation is shown in Fig. 12.

The association of three strands of DNA increases the negative charge density, and hence a higher level of positive charges is required to stabilize the triple helix. In general, triplex DNA is stabilized by cation concentrations comparable to or higher than their physiological level. For example, 150 m*M* NaCl or 25 m*M* $MgCl_2$ can stabilize triplex structures in poly(dA)/2poly(dT). Hampel et al. *(47)* demonstrated that triplexes could be formed at the physiological pH of 7.0 in the presence of physiological concentrations (0.5–1 m*M*) of spermine. This stabilizing effect of polyamines was attributed to the changes in the overall charge density of the triplex DNA brought about by polyamine–DNA interactions. Initially, the triplex may bind with polyamines more strongly than the duplex because of a higher negative charge of the former, resulting in a shift of the equilibrium in favor of triplex formation.

Thomas and Thomas *(48)* questioned whether polyamine structure played a role in triplex DNA stabilization. To address this problem, they conducted T_m measurements of poly(dA)/2poly(dT) triplex in the presence of putrescine, spermidine, spermine, and homologs of putrescine and spermidine (Figs. 13 and 14). In the presence of polyamines, the absorbance vs temperature profile showed two transitions: T_{m1}, corresponding to triplex $\rightarrow$ duplex+single-stranded DNA, and T_{m2}, corresponding to duplex melting.

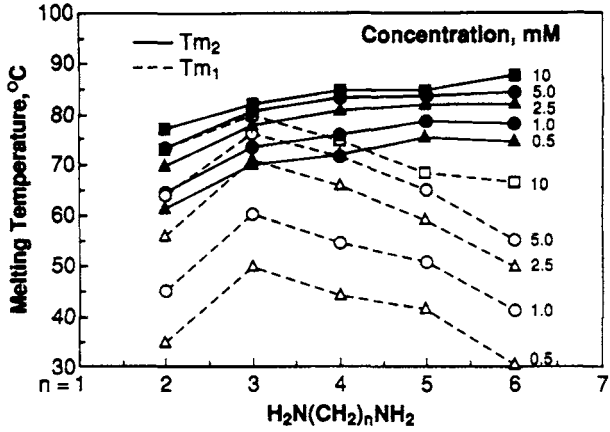

Fig. 13. Structural specificity effects of putrescine homologs on the melting temperature of triplex (T_{m1}) and duplex (T_{m2}) forms of DNA. The number of methylene groups (*n*) in putrescine homologs is plotted against the melting temperature at different diamine concentrations. (Reprinted with permission from ref. *48*. © 1993 American Chemical Society.)

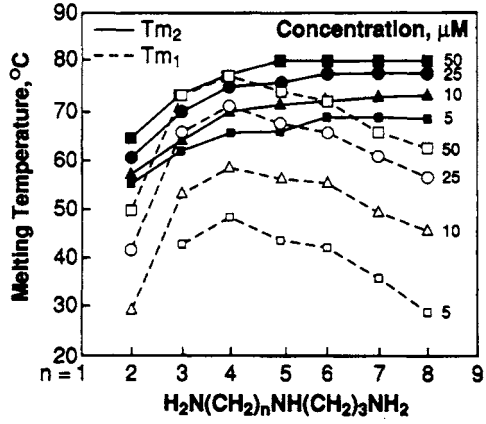

Fig. 14. Structural specificity effects of spermidine homologs on the melting temperature of triplex (T_{m1}) and duplex (T_{m2}) forms of DNA. The number of methylene groups (*n*) in putrescine homologs is plotted against the melting temperature at different diamine concentrations. (Reprinted with permission from ref. *48*. © 1993 American Chemical Society.)

Figures 13 and 14 show the structural specificity of the various analogs, which vary in the number of methylene ($–CH_2–$) groups, plotted against the T_m. In the presence of 0.5 m*M* putrescine, T_{m1} and T_{m2} were 44.8° and 71°C, respectively, in 10 m*M* Na cacodylate buffer. With 2.5 µ*M* spermidine or 0.1 µ*M* spermine, T_{m1} values were 42.8°C and 54.4°C and T_{m2} values were 65 and 82°C, respectively. These results showed that the ability of natural polyamines to stabilize the triplex DNA increased in the following order: spermine > spermidine > putrescine. In a series of putrescine homologs,

$H_2N(CH_2)_nNH_2$ (in which $n = 2–6$; $n = 4$ for putrescine), diaminopropane ($n = 3$) was the most effective stabilizer of triplex DNA. Among a series of triamines $H_2N(CH_2)_3$-$NH(CH_2)_nNH_2$ (in which $n = 2–8$; $n = 4$ for spermidine), spermidine was the most effective triplex stabilizing agent. Similar structural specificity was found with spermine homologs, $H_2N(CH_2)_3NH(CH_2)_nNH(CH_2)_3NH_2$ (in which $n = 2–12$; $n = 4$ for spermine). Interestingly, effects of these homologs on the poly(dA)/poly(dT) duplex were relatively insensitive to changes in the length of the methylene-bridging region. Differential effects of polyamine analogs in stabilizing triplex vs duplex DNA suggested their potential application in triplex DNA-based antigene therapeutics.

Effects of polyamines on the stabilization of triplex DNA formed from a purine motif triplex-forming oligonucleotide, 5′-TG3TG4TG4TG3T-3′, and its target duplex probe, consisting of the oligonucleotides 5′-TCGAAG3AG4AG4AG3A-3′ and 5′-TCGATC3TC4TC4TC3T-3′, were studied in the presence of natural and synthetic polyamines *(49)*. Electrophoretic mobility shift assay showed that *bis*(ethyl) analogs of spermine and its higher analog, 4-4-4-4 were excellent stabilizers of triplex DNA (Fig. 15). In contrast, the non-*bis*(ethyl) substituted parent polyamines aggregated the oligonucleotides in preference to triplex DNA stabilization. Temperature-dependent circular dichroism (CD) spectra of triplex DNA showed monophasic melting transition in the absence and presence of polyamines, suggesting duplex/triplex single-stranded DNA transition. These results indicate that structural modifications of polyamines are an effective strategy to develop triplex DNA-stabilizing ligands, with potential applications in antigene therapeutics. For example, diaminopropane stabilized triplex DNA and suppressed the *c-myc* oncogene expression in MCF-7 breast cancer cells at a level higher than that of controls in the absence of the diamine *(50)*.

8. DNA Nanoparticle Formation, Aggregation, and Resolubilization

DNA condensation to nanoparticles is a process in which a significant decrease in the total volume occupied by the DNA occurs, with an orderly collapse of DNA to compact particles of finite size and orderly morphology *(13)*. Condensation is generally provoked under conditions of low concentrations (low micromolar) of DNA and multivalent cations, such as polyamines, inorganic cations (cobalt hexamine ($Co(NH_3)_6^{3+}$), and polymers, although the presence of dehydrating solvents can accelerate this process by a change in the dielectric constant of the medium *(51–53)*. Condensing agents usually work by reducing the repulsions between DNA segments by neutralizing the negative phosphate charges or by reorienting water dipoles near DNA surfaces. In the condensed DNA, just one or two layers of water may separate the helices. DNA condensation is an example of polymer–globule transition, and under appropriate conditions of polymer length and stiffness, it is a readily reversible process. DNA condensation is biologically significant, as it appears to be the method by which DNA is packaged into viral heads. Recent research indicates that DNA condensation is a prerequisite for the transport of DNA through the cell membrane for gene therapy applications.

Binding of polyamines to DNA results in the localized bending or distortion of DNA and the facilitation of DNA collapse to rods, spheres, and toroids. In 1976, Gosule and

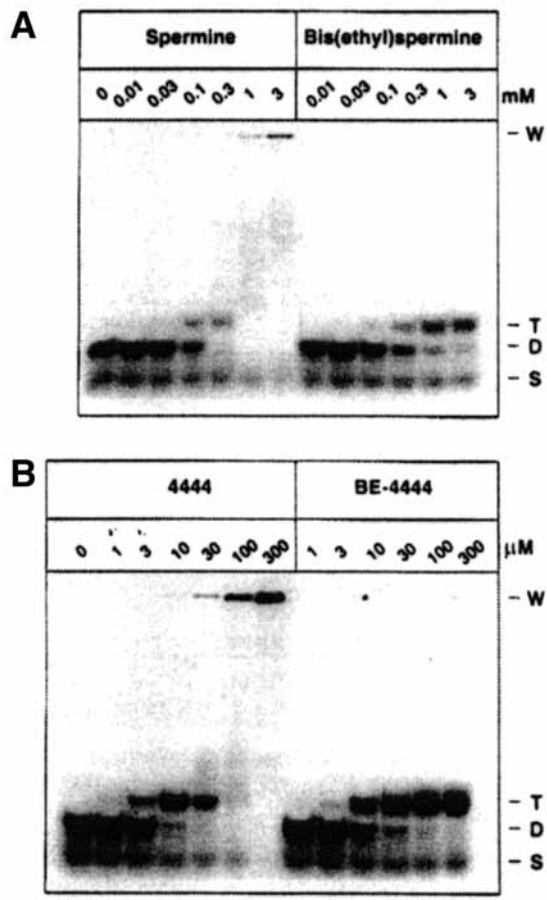

Fig. 15. Facilitation of purine-motif triplex formation by different polyamines. Shown are electrophoretic mobility shift assays of triplex formation by the oligonucleotide ODN1 in the presence of different polyamine concentrations. D, duplex DNA; S, single-stranded probe; T, triplex; W, probe DNA retained in the polyacrylamide gel well. **(A)** Spermine and *bis*(ethyl)spermine. **(B)** 4444 and BE-4444. (Reprinted with permission from ref. *49*. © 1997 American Chemical Society.)

Schellman *(52)* reported that the addition of spermidine to DNA resulted in the formation of small, roughly spherical particles. Electron microscopic examination showed the presence of rods, toroids, and spheroids when DNA was condensed by polyamines. Wilson and Bloomfield *(53)* applied the counterion condensation theory to calculate the amount of charge neutralization necessary to collapse DNA to nanoparticles, and found that 89–90% of the negative phosphate charges are neutralized by spermidine and spermine in aqueous medium. They also found that at least a trivalent cation is required for DNA condensation, and that mono and divalent ions effectively competed with multivalent ions to prevent DNA condensation or decondense compacted DNA.

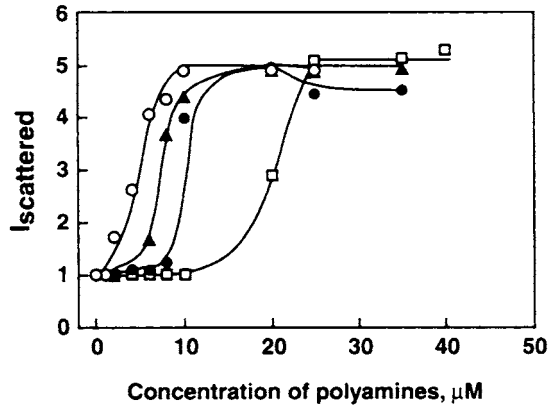

Fig. 16. Typical plots of the relative intensity of scattered light at 90° plotted against the concentrations of spermine (o), 3–10–3 (▲), 3–11–3 (●), and 3–12–3 (□). The λ-DNA solution had a concentration of 1.5 μ*M* DNA phosphate, dissolved in 10 m*M* sodium cacodylate buffer, pH 7.4. (Reprinted with permission from ref. *56*. © 2001 American Chemical Society.)

Allison et al. *(54)* first reported structural specificity effects of polyamines on DNA condensation. They investigated the ability of spermidine homologs to provoke DNA condensation and found significant structural effects, in addition to the general electrostatic effects. For example, a higher homolog of spermidine, aminopropyl diaminoheptane, induced aggregation of DNA rather than monomolecular collapse. Thomas and Bloomfield *(55)* further found that $Co(NH_3)_6^{3+}$ was more effective than isovalent spermidine^{3+} in causing DNA condensation at pH values between 5.1 and 10.2. In addition, DNA nanoparticles produced by $Co(NH_3)_6^{3+}$ were smaller than those obtained with spermidine. Vijayanathan et al. *(56)* determined the structural effects of tetravalent spermine homologs on DNA condensation and found that lower homologs of spermine were more efficacious in condensing DNA than the higher homologs (Fig. 16). Hydrodynamic radii (R_h) of DNA condensates increased with the increase in the length of methylene bridging in the series, $H_2NH(CH_2)_3NH(CH_2)_nNH(CH_2)_3NH_2$, where n = 2–12 (*see* Fig. 1 for chemical structures and abbreviations). Condensates formed in the presence of spermine and its nearest homologs (3-3-3, 3-5-3, and 3-6-3) exhibited hydrodynamic radii (R_h) between 40 and 50 nm. The R_h values, however, increased to 60–70 nm for 3-7-3, 3-8-3, 3-9-3, and 3-10-3, and greater than 100 nm for 3-11-3 and 3-12-3 (Table 3). Thus they found that the relative order of spermine homologs in condensing DNA to be in the following order: 3-6-3 < 3-5-3 ≈ 3-4-3 < 3-7-3 ≈ 3-8-3 < 3-9-3 < 3-10-3 ≈ 3-3-3 << 3-2-3 ≈ 3-11-3 << 3-12-3. Toroidal condensates were observed with atomic force microscopy in aqueous solution, when a plasmid DNA was treated with spermine and its higher analogs (Fig. 17) *(57)*. The structural effects of polyamines on R_h values were also evident in the temperature-dependent stabilization of nanoparticles *(58)*. These results confirm that there are specific ion effects in DNA condensation by oligocations.

DNA condensation involves the collapse of one or a few molecules of DNA to morphologically distinct nanoparticles. This process is different from aggregation, which

Table 3
Hydrodynamic Properties of DNA Condensates Formed in the Presence of Spermine and Its Homologs

Polyamine homolog	Diffusion coefficient (cm^2/s)	Hydrodynamic radius[a] (nm)
3-3-3	4.5×10^{-8}	50
3-4-3 (spermine)	5.5×10^{-8}	41
3-5-3	4.7×10^{-8}	50
3-6-3	4.8×10^{-8}	48
3-7-3	4.0×10^{-8}	59
3-8-3	3.6×10^{-8}	63
3-9-3	3.4×10^{-8}	67
3-10-3	3.3×10^{-8}	69
3-11-3	2.4×10^{-8}	101
3-12-3	1.7×10^{-8}	132

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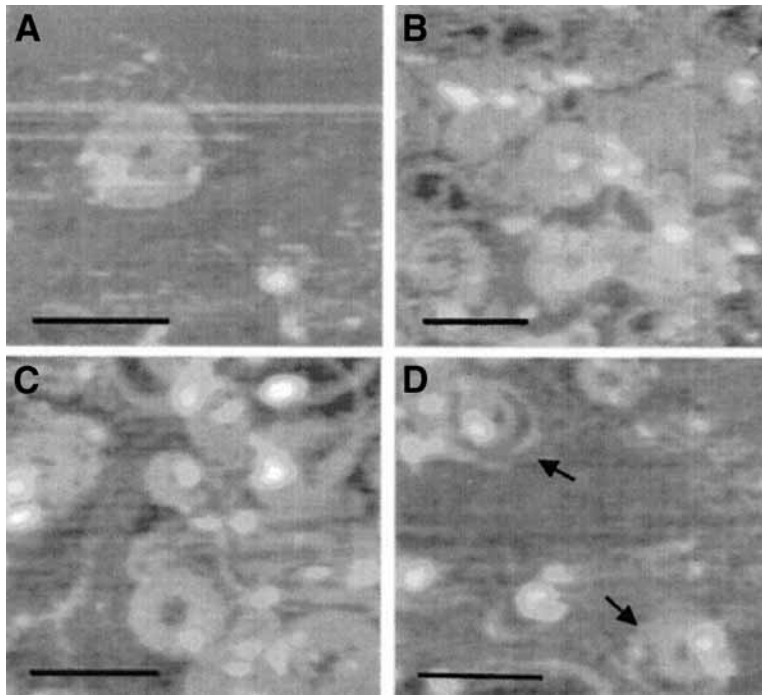

Fig. 17. Scanning force microscopy images showing the toroid structures of pGL3 plasmid DNA formed by incubation with 25 μ*M* spermine **(A)**, 5 μ*M* 3-3-3-3 **(B)**, 2 μ*M* 3-4-3-4-3 (**C**), and the partly formed toroids, observed in the presence of 2 μ*M* 3-4-3-4-3 (**D**). Scale bar is 200 nm. (Reprinted with permission from ref. *57*.)

involves several molecules of DNA to produce bundles or amorphous precipitates. DNA condensation and aggregation are related phenomena and are governed by similar structural specificity effects. Schellman and Parthasarathy *(59)* demonstrated that the structural arrangement of DNA collapsed by spermidine homologs depended on the chemical structure of the polyamines. They used the same series of spermidine homologs, $H_2N(CH_2)_3NH(CH_2)_nNH_2$, that were used by Thomas and Bloomfield *(44)* for T_m measurements and Allison et al. *(54)* for condensation experiments. The Bragg spacing and the interhelical spacing for a hexagonal packing model for DNA varied systematically with the length of the methylene bridge of the homologs. Pelta et al. *(60)* showed that at high concentrations (~m*M*), aggregates of calf thymus DNA could assume a liquid crystalline state in the presence of spermidine and spermine. Hexagonal packaging of DNA was found in liquid crystalline textures formed by polyamine–DNA interactions. Structural specificity effects of polyamines were also evident in the stabilization of liquid crystalline textures of DNA (Fig. 18) *(61)*.

Polyamine structural dependence on triplex DNA aggregation was studied by Musso et al. *(47)*. They found that *bis*(ethyl) substitution of polyamines decreased the ability of the parent compound to aggregate DNA. Triplex DNA was more labile to undergo aggregation than double helical DNA. For example, the ability of spermine to provoke DNA precipitation was in the following order: triplex DNA > duplex DNA > single-stranded DNA. The effective concentration of spermine to precipitate DNA increased with Na^+ in the medium. An interesting observation related to the aggregation of DNA was the resolubilization of the aggregates at millimolar concentrations of polyamines (Fig. 19). T_m data indicated that polyamines stabilized DNA even in the resolubilization state. Chemical structural specificity of spermidine and spermine analogs were observed in the resolubilization of sonicated calf thymus DNA, with N^4-methyl substitution of spermidine and a heptamethylene separation of the imino groups of spermine exerting the maximal difference in the precipitating DNA compared with spermidine and spermine, respectively.

Pelta et al. *(60)* studied the precipitation of short DNA molecules by spermidine, spermine, and cobalt hexamine, and found that the addition of these cations to a DNA solution led first to the precipitation of the DNA, and further addition led to resolubilization of the DNA pellet. The multivalent salt concentration required for resolubilization is essentially independent of the DNA concentration (between 1 μg/mL and 1 mg/mL) and of the monovalent cation concentration (up to 100 m*M*) present in the DNA solution. Saminathan et al. *(62)* further showed that the aggregation and resolubilization of DNA by polyamines are essentially independent of the length of the DNA. CD spectroscopy revealed a series of sequential conformational alterations of duplex and triplex DNA, with the duplex form regaining the B-DNA conformation at high concentrations (approx 200 m*M*) of spermine (Fig. 20). The triplex DNA, however, remained in a Ψ-DNA conformation in the resolubilization state. The Ψ-DNA form is believed to be a tightly packaged, twisted liquid crystalline form of DNA. The CD spectrum of Ψ-DNA arises from the interaction of circularly polarized light with the highly ordered tertiary structure, which depends on adjacent superhelical turns. Although base sequence specificity of polyamine-induced DNA condensation and aggregation has not been well-characterized, CD studies and centrifugation studies of an oligonucleotide harboring

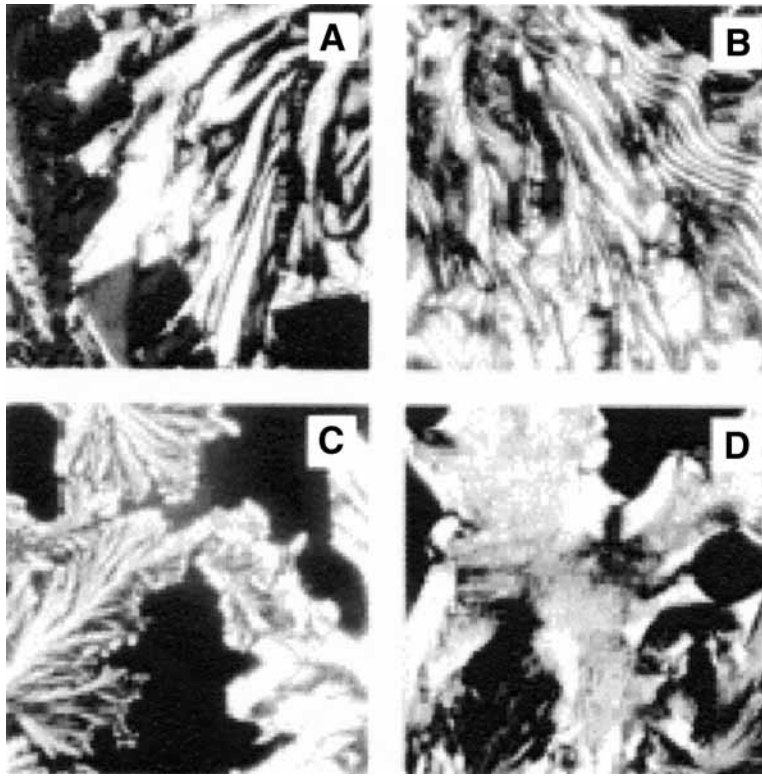

Fig. 18. Effects of higher homologs of spermine on the liquid crystalline phase transitions of calf thymus DNA. **(A)** DNA (25 m*M* in Na cacodylate buffer) was incubated with 1 m*M* 3-5-3 for 24 h at 37°C. A crystalline phase is observed (×100). **(B)** A myelin-like growth, which developed a striped appearance, is found after incubation of DNA with 1 m*M* 3–6–3 for 12 h at 37°C (×360). **(C)** A crystalline phase was observed on further incubating the DNA in (**B**) for 24 h at 37°C (×90). **(D)** Fingerprint textures are obtained on incubating DNA with 1 m*M* 3-9-3 for 12 h at 37°C (×200). (Reprinted with permission from ref. *61*.)

estrogen response element (ERE) and control sequences indicated that the sequence containing ERE is more prone to conformational transitions and aggregation (*63*,*64*). Thus, the first step in DNA condensation and aggregation appears to be a high-affinity, site-specific interaction between polyamines and DNA, followed by multisite interaction. Multiple sites of interaction can occur around a nucleation center in a single, long DNA molecule, resulting in toroid formation at low concentrations of DNA or a unique ordering of multiple molecules in the case of oligonucleotides.

9. Theories of Polyamine–DNA Binding

The exact mechanism of polyamine binding to DNA is a contentious issue. Some researchers believe that polyamine–DNA interaction is primarily electrostatic in

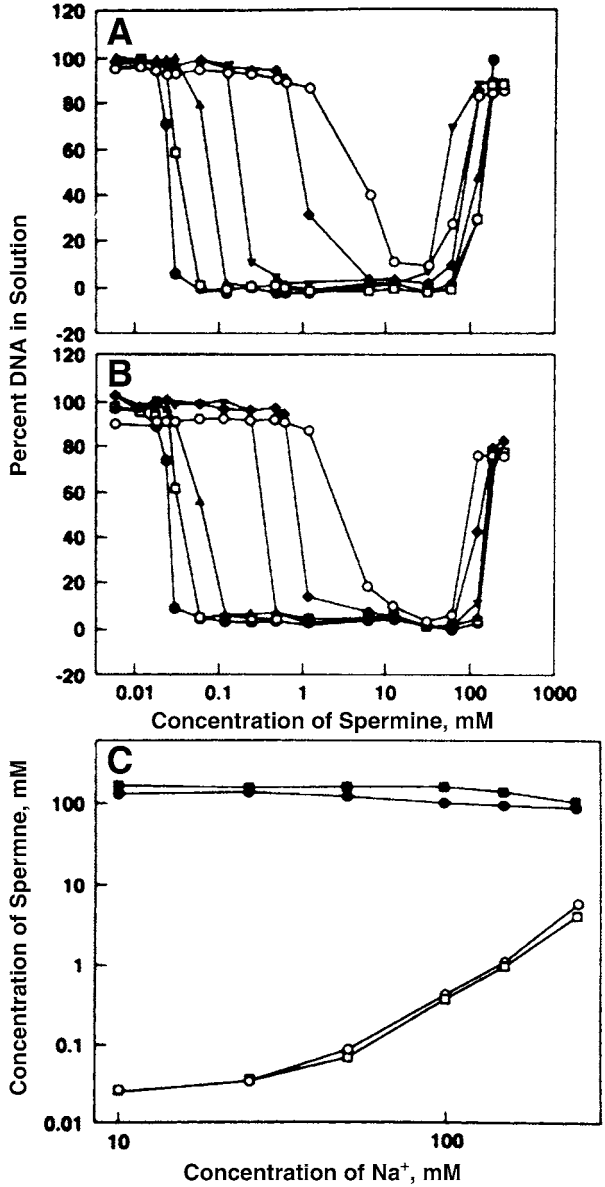

Fig. 19. Precipitation/resolubilization of sonicated **(A)** and native **(B)** calf thymus DNA by spermine in the presence of 10 (●), 25 (□), 50 (▲), 100 (▼), 150 (◆), and 250 (○) m*M* Na^+. A logarithmic scale is used for spermine concentrations. Experiments were performed in 10 m*M* cacodylate buffer (pH 7.4). Higher concentrations of Na^+ were achieved by adding small volumes of 4 *M* NaCl solution. **(C)** Concentrations of spermine required for 50% DNA precipitation (open symbols) and resolubilization (filled symbols) of sonicated (○,●) and native (□,■) calf thymus DNA. Logarithmic scale is used for both *x* and *y* axes of **(C)**. (Reprinted with permission from ref. 62. © 1999 American Chemical Society.)

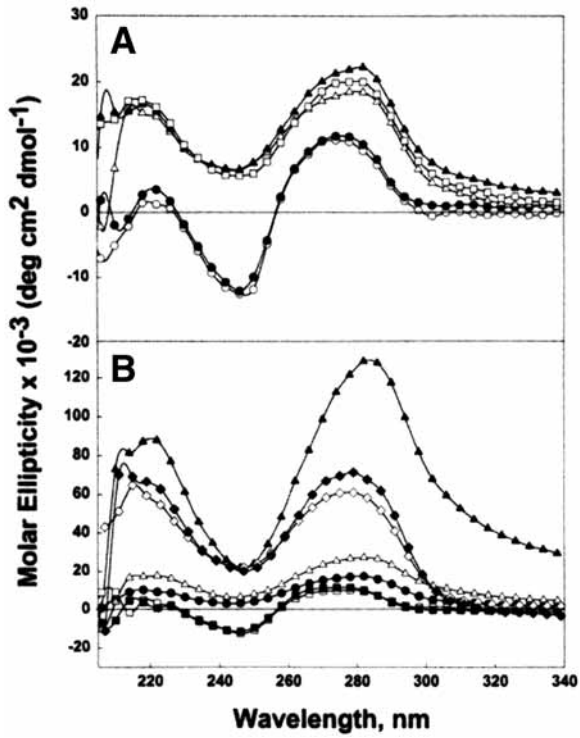

Fig. 20. Circular dichroism (CD) spectra of sonicated calf thymus DNA in the presence of spermine in a buffer containing 10 m*M* sodium cacodylate, 0.5 m*M* EDTA, pH 7.4. Concentrations of spermine are as follows. **(A)** 0 (o), 0.015 (●), 0.03 (Δ), 0.05 (▲), and 0.10 (□) m*M*; **(B)** 25 (●), 75 (Δ), 90 (◇), 100 (▲), 110 (◆), 125 (□), and 200 (n) m*M*. Because the solutions were not centrifuged, **(B)** shows the CD spectra of either aggregated DNA (●,Δ) or resolubilized DNA (▲,◆,□,■). (Reprinted with permission from ref. *62*. © 1999 American Chemical Society.)

nature *(53)*. However, other studies show that polyamine–DNA binding cannot be solely explained by the electrolytic theory and that structural specificities have to be considered to explain the true nature of polyamine–DNA binding *(13)*.

The nonspecific electrostatic interaction theory of polyamine–DNA binding is supported by the polyelectrolyte and counterion condensation theories developed by Manning *(65)*. According to these, the positively charged polyamines are structureless point charges that form an ionic cloud near the DNA surface from nonspecific electrostatic interactions with the negative phosphate groups of DNA. Site-specific interactions between polyamines and DNA are not considered in these models. This line of thought has been given credence by results from earlier solution studies that used equilibrium dialysis, nuclear magnetic resonance measurements on ^{23}Na, ^{14}N, and ^{1}H nuclei, and calorimetric methods to study polyamine–DNA binding (*13,66*). However, evidence has been accumulating that suggest that the sequence and structure of DNA plays a significant role in determining polyamine–DNA binding. Site-specific interaction of

polyamines with DNA is exemplified by preferential binding of polyamines with A-DNA and Z-DNA.

Experimental and theoretical methods have been used to determine the binding positions of polyamines on DNA. Most of these studies have focused on spermine, as it is often used to crystallize DNA from solution. Different and often contradictory ideas concerning the binding sites of spermine and other polyamines to DNA have been proposed. Energy minimization calculations using the $(dG\text{-}dC)_5/(dG\text{-}dC)_5$ model suggest the folding of the major groove of DNA around spermine with the widening of the minor groove and compaction of the intrastrand phosphate distances *(33)*. Recent molecular modeling studies using a B-DNA model indicate that spermine binding is highly mobile with binding sites across major and minor grooves and around the phosphates, and is capable of forming bridges between different helices *(32)*. Rapid diffusion of spermine along the DNA duplex with specific tight-binding sites or delocalized binding with no discrete sites has been suggested. Infrared studies agree with the modeling studies, demonstrating that there could be interstrand attachment at both the major and minor grooves, although the major groove is the preferred binding site of spermine *(67)*. Raman spectroscopic studies by different investigators have indicated base-specific, as well as electrostatic interactions, independent of base composition (*68*,*69*). Thus, the binding positions of spermine and other polyamines appears to be unresolved and it is possible that the extent of spermine binding and its exchange with other cations and the hydration shell at any particular site depends on several parameters, including DNA sequence, geometry of grooves, and the nature of other cations and anions in the medium. The requirement of polyamines in the function of normal cells and their increased biosynthesis in diseases, such as cancer, has led to the development of synthetic polyamine analogs *(70)*. Therefore, it is important to know how natural polyamines and synthetic analogs interact with nucleic acids.

10. Future Directions

The role of polyamines in the function of DNA is multifaceted. As polyamines bind to DNA, DNA conformation changes and therefore DNA binding affinity of gene regulatory proteins is modulated. Certain DNA conformational variations enhance protein binding, whereas others inhibit protein–DNA interactions. Certain protein-binding sites might be masked by polyamines, which will be unmasked by acetylation of polyamines, relieving polyamine interactions with DNA. Thus, the binding of polyamines to DNA introduces a dynamic role in gene expression as polyamine levels are exquisitely regulated by biosynthetic and catabolic enzymes and by uptake/efflux pathways. These direct effects of polyamines on gene regulation will be in addition to the effects of polyamines on cell signaling pathways and kinase activities. Thus there are numerous opportunities that need to be explored in controlling gene expression, cell-cycle regulation, and malignant cell growth by interfering with cellular regulation of polyamines.

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References

1. Thomas, T. and Thomas, T. J. (2003) Polyamine metabolism and cancer. *J. Cell. Mol. Med.* **7,** 113–126.
2. Huang, Y., Pledgie, A., Casero, R. A. Jr., and Davidson, N. E. (2005) Molecular mechanisms of polyamine analogs in cancer cells. *Anticancer Drugs* **16,** 229–241.
3. Igarashi, K. and Kashiwagi, K. (2000) Polyamines: mysterious modulators of cellular functions. *Biochem. Biophys. Res. Commun.* **271,** 559–564.
4. Childs, A. C., Mehta, D. J., and Gerner, E. W. (2003) Polyamine-dependent gene expression. *Cell. Mol. Life Sci.* **60,** 1394–1406.
5. Thomas, T. and Kiang, D. T. (1988) A twenty-two-fold increase in the relative affinity of estrogen receptor to poly (dA-dC).poly (dG-dT) in the presence of polyamines. *Nucleic Acids Res.* **16,** 4705–4720.
6. Shah, N., Thomas, T. J., Lewis, J. S., et al. (2001) Regulation of estrogenic and nuclear factor kappa B functions by polyamines and their role in polyamine analog-induced apoptosis of breast cancer cells. *Oncogene* **20,** 1715–1729.
7. Chattopadhyay, M. K., Tabor, C. W., and Tabor, H. (2003) Polyamines protect Escherichia coli cells from the toxic effect of oxygen. *Proc. Natl. Acad. Sci. USA.* **100,** 2261–2265.
8. Thomas, T. and Thomas, T. J. (2001) Polyamines in cell growth and cell death: molecular mechanisms and therapeutic applications. *Cell. Mol. Life Sci.* **58,** 244–258.
9. Tabor, C. W. and Tabor, H. (1984) Polyamines. *Annu. Rev. Biochem.* **53,** 749–790.
10. Xaplanteri, M. A., Petropoulos, A. D., Dinos, G. P., and Kalpaxis, D. L. (2005) Localization of spermine binding sites in 23S rRNA by photoaffinity labeling: parsing the spermine contribution to ribosomal 50S subunit functions. *Nucleic Acids Res.* **33,** 2792–2805.
11. Jovine, L., Djordjevic, S., and Rhodes, D. (2000) The crystal structure of yeast phenylalanine tRNA at 2.0 A resolution: cleavage by Mg(2+) in 15-year old crystals. *J. Mol. Biol.* **301,** 401–414.
12. Venkiteswaran, S., Vijayanathan, V., Shirahata, A., Thomas, T., and Thomas, T. J. (2005) Antisense recognition of the HER-2 mRNA: effects of phosphorothioate substitution and polyamines on DNA.RNA, RNA.RNA, and DNA.DNA duplex stability. *Biochemistry* **44,** 303–312.
13. Vijayanathan, V., Thomas, T., and Thomas, T. J. (2002) DNA nanoparticles and development of DNA delivery vehicles for gene therapy. *Biochemistry* **41,** 14,085–14,094.
14. Basu, H. S., Smirnov, I. V., Peng, H. F., Tiffany, K., and Jackson, V. (1997) Effects of spermine and its cytotoxic analogs on nucleosome formation on topologically stressed DNA in vitro. *Eur. J. Biochem.* **243,** 247–258.
15. Wahl, M. C. and Sundaralingam, M. (1995) New crystal structures of nucleic acids and their complexes. *Curr. Opin. Struct Biol.* **5,** 282–295.
16. Herbert, A. and Rich, A. (1996) The biology of left handed Z-DNA. *J. Biol. Chem.* **271,** 11,595–11,598.
17. Minyat, E. E., Ivanov, V. I., Kritzyn, A. M., Minchenkova, L. E., and Schyolkina, A. K. (1979) Spermine and spermidine-induced B to A transition of DNA in solution. *J. Mol. Biol.* **128,** 397–409.
18. Zakrzewska, K. and Pullman, B. (1986) Spermine-nucleic acid interactions: a theoretical study. *Biopolymers* **25,** 375–392.
19. Sy, D., Hugot, S., Savoye, C., Ruiz, S., Charlier, M., and Spotheim-Maurizot, M. (1999) Radioprotection of DNA by spermine: a molecular modelling approach. *Int. J. Radiat. Biol.* **75,** 953–961.

20. Suwalsky, M., Traub, W., Shmueli, U., and Subirana, J. A. (1969) An X-ray study of the interaction of DNA with spermine. *J. Mol. Biol.* **42,** 363–373.
21. Feuerstein, B. G., Pattabiraman, N., and Marton, L. J. (1989) Molecular dynamics of spermine-DNA interactions: sequence specificity and DNA bending for a simple ligand. *Nucleic Acids Res.* **17,** 6883–6892.
22. Jain, S., Zon, G., and Sundaralingam, M. (1989) Base only binding of spermine in the deep groove of the A-DNA octamer d(GTGTACAC). *Biochemistry* **28,** 2360–2364.
23. Tippin, D. B. and Sundaralingam, M. (1997) Nine polymorphic crystal structures of d(CCGGGCCCGG), d(CCGGGCCm5CGG), d(Cm5CGGGCCm5CGG) and d(CCGGGCC(Br)5CGG) in three different conformations: effects of spermine binding and methylation on the bending and condensation of A-DNA. *J. Mol. Biol.* **267,** 1171–1185.
24. Tari, L. W. and Secco, A. S. (1995) Base-pair opening and spermine binding—B-DNA features displayed in the crystal structure of a gal operon fragment: implications for protein-DNA recognition. *Nucleic Acids Res.* **23,** 2065–2073.
25. Lindemose, S., Nielsen, P. E., and Mollegaard, N. E. (2005) Polyamines preferentially interact with bent adenine tracts in double-stranded DNA. *Nucleic Acids Res.* **33,** 1790–1803.
26. Xiao, L., Swank, R. A., and Matthews, H. R. (1991) Photoaffinity polyamines: sequence-specific interactions with DNA. *Nucleic Acids Res.* **19,** 3701–3708.
27. Marquet, R. and Houssier, C. (1988) Different binding modes of spermine to A-T and G-C base pairs modulate the bending and stiffening of the DNA double helix. *Biochem. Pharmacol.* **37,** 1857–1858.
28. Basu, H. S., Shafer, R. H., and Marton, L. J. (1987) A stopped-flow H-D exchange kinetic study of spermine-polynucleotide interactions. *Nucleic Acids Res.* **15,** 5873–5886.
29. Plum, G. E. and Bloomfield, V. A. (1990) Structural and electrostatic effects on binding of trivalent cations to double-stranded and single-stranded poly[d (AT)]. *Biopolymers* **29,** 13–27.
30. Behe, M. and Felsenfeld, G. (1981) Effects of methylation on a synthetic polynucleotide: the B-Z transition in poly(dG-m5dC).poly(dG-m5dC). *Proc. Natl. Acad. Sci. USA* **78,** 1619–1623.
31. Howell, M. L., Schroth, G. P., and Ho, P. S. (1996) Sequence-dependent effects of spermine on the thermodynamics of the B-DNA to Z-DNA transition. *Biochemistry* **35,** 15,373–15,382.
32. Korolev, N., Lyubartsev, A. P., Nordenskiold, L., and Laaksonen. A. (2001) Spermine: an "invisible" component in the crystals of B-DNA. A grand canonical Monte Carlo and molecular dynamics simulation study. *J. Mol. Biol.* **308,** 907–917.
33. Feuerstein, B. G., Pattabiraman, N., and Marton, L. J. (1986) Spermine-DNA interactions: a theoretical study. *Proc. Natl. Acad. Sci. USA* **83,** 5948–5952.
34. Ohishi, H., Suzuki, K., Ohtsuchi, M., Hakoshima, T., and Rich, A. (2002) The crystal structure of N(1)-[2-(2-amino-ethylamino)-ethyl]-ethane-1,2-diamine (polyamines) binding to the minor groove of d(CGCGCG)(2), hexamer at room temperature. *FEBS Lett.* **523,** 29–34.
35. Thomas, T. J. and Messner, R. P. (1988) Structural specificity of polyamines in left-handed Z-DNA formation. Immunological and spectroscopic studies. *J. Mol. Biol.* **201,** 463–467.
36. Thomas, T. J., Gunnia, U. B., and Thomas T. (1991) Polyamine-induced B-DNA to Z-DNA conformational transition of a plasmid DNA with $(dG\text{-}dC)_n$ insert. *J. Biol. Chem.* **266,** 6137–6141.
37. Geiger, L. E. and Morris, D. R. (1978) Polyamine deficiency reduces the rate of DNA replication fork movement in Escherichia coli. *Nature* **272,** 730–732.
38. Porter, C. W. and Bergeron, R. J. (1983) Spermidine requirement for cell proliferation in eukaryotic cells: structural specificity and quantitation. *Science* **219,** 1083–1085.

39. Panagiotidis, C. A., Artandi, S., Calame, K., and Silverstein, S. J. (1995) Polyamines alter sequence-specific DNA-protein interactions. *Nucleic Acids Res.* **23,** 1800–1809.
40. Kuramoto, N., Inoue, K., Takano, K., et al. (2003) A possible novel mechanism underlying temperature-dependent uptake of [^{3}H]spermidine in nuclear fractions of murine brain. *Brain Res.* **981,** 78–84.
41. Lewis, J. S., Vijayanathan, V., Thomas, T. J., et al. (2005) Activation of cyclin D1 by estradiol and spermine in MCF-7 breast cancer cells; A mechanism involving the p38 MAP kinase and phosphorylation of ATF-2. *Oncol. Res.* **15,** 113–128.
42. Tabet, M., Labroo, V., Sheppard, P., and Sasaki, T. (1993) Spermine-induced conformational changes of a synthetic peptide. *J. Am. Chem. Soc.* **115,** 3866–3868.
43. Tabor, H. (1962) The protective effect of spermine and other polyamines against heat denaturation of deoxyribonucleic acid. *Biochemistry* **1,** 496–501.
44. Thomas, T. J. and Bloomfield, V. A. (1984) Ionic and structural effects on the thermal helix-coil transition of DNA complexed with natural and synthetic polyamines. *Biopolymers* **23,** 1295–1306.
45. Braunlin, W. H., Strick, T. J., and Record, M. T., Jr. (1982) Equilibrium dialysis studies of polyamine binding to DNA. *Biopolymers* **21,** 1301–1314.
46. Uil, T. G., Haisma, H. J., and Rots, M. G. (2003) Therapeutic modulation of endogenous gene function by agents with designed DNA-sequence specificities. *Nucleic Acids Res.* **31,** 6064–6078.
47. Hampel, K. J., Crosson, P., and Lee, J. S. (1991) Polyamines favor DNA triplex formation at neutral pH. *Biochemistry* **30,** 4455–4459.
48. Thomas, T. and Thomas, T. J. (1993) Selectivity of polyamines in triplex DNA stabilization. *Biochemistry* **32,** 14,068–14,074.
49. Musso, M., Thomas, T., Shirahata, A., Sigal, L. H., Van Dyke, M. W., and Thomas, T. J. (1997) Effects of chain length modification and bis(ethyl) substitution of spermine analogs on purine-purine-pyrimidine triplex DNA stabilization, aggregation, and conformational transitions. *Biochemistry* **36,** 1441–1449.
50. Thomas, T. J., Faaland, C. A., Gallo, M. A., and Thomas T. (1995) Suppression of c-myc oncogene expression by a polyamine-complexed triplex forming oligonucleotide in MCF-7 breast cancer cells. *Nucleic Acids Res.* **23,** 3594–3599.
51. Raspaud, E., Durand, D., and Livolant, F. (2005) Interhelical spacing in liquid crystalline spermine and spermidine-DNA precipitates. *Biophys. J.* **88,** 392–403.
52. Gosule, L. C. and Schellman, J. A. (1976) Compact form of DNA induced by spermidine. *Nature* **259,** 333–335.
53. Wilson, R. W. and Bloomfield, V. A. (1979) Counterion-induced condensation of deoxyribonucleic acid. a light-scattering study. *Biochemistry* **18,** 2192–2196.
54. Allison, S. A., Herr, J. C., and Schurr, J. M. (1981) Structure of viral phi 29 DNA condensed by simple triamines: a light scattering and electron-microscopy study. *Biopolymers* **20,** 469–488.
55. Thomas, T. J. and Bloomfield, V. A. (1983) Collapse of DNA caused by trivalent cations: pH and ionic specificity effects. *Biopolymers* **22,** 1097–1106.
56. Vijayanathan, V., Thomas, T., Shirahata, A., and Thomas, T. J. (2001) DNA condensation by polyamines: a laser light scattering study of structural effects. *Biochemistry* **40,** 13,644–13,651.
57. Vijayanathan, V., Thomas, T., Antony, T., Shirahata, A., and Thomas, T. J. (2004) Formation of DNA nanoparticles in the presence of novel polyamine analogues: a laser light scattering and atomic force microscopic study. *Nucleic Acids Res.* **32,** 127–134.

58. Vijayanathan, V., Lyall, J., Thomas, T., Shirahata, A., and Thomas, T. J. (2005) Ionic, structural, and temperature effects on DNA nanoparticles formed by natural and synthetic polyamines. *Biomacromolecules* **6,** 1097–1103.
59. Schellman, J. A. and Parthasarathy, N. (1984) X-ray diffraction studies on cation-collapsed DNA. *J. Mol. Biol.* **175,** 313–329.
60. Pelta, J. Jr., Durand, D., Doucet, J., and Livolant, F. (1996) DNA mesophases induced by spermidine: structural properties and biological implications. *Biophys. J.* **71,** 48–63.
61. Saminathan, M., Thomas, T., Shirahata, A., Pillai, C. K., and Thomas, T. J. (2002) Polyamine structural effects on the induction and stabilization of liquid crystalline DNA: potential applications to DNA packaging, gene therapy and polyamine therapeutics. *Nucleic Acids Res.* **30,** 3722–3731.
62. Saminathan, M., Antony, T., Shirahata, A., Sigal, L. H., Thomas, T., and Thomas, T. J. (1999) Ionic and structural specificity effects of natural and synthetic polyamines on the aggregation and resolubilization of single-, double-, and triple-stranded DNA. *Biochemistry* **38,** 3821–3830.
63. Lewis, J. S., Thomas, T. J., Shirahata, A., and Thomas T. (2000) Self-assembly of an oligodeoxyribonucleotide harboring the estrogen response element in the presence of polyamines: ionic, structural, and DNA sequence specificity effects. *Biomacromolecules* **1,** 339–349.
64. Thomas, T., Kulkarni, G. D., Gallo, M. A., et al. (1997) Effects of natural and synthetic polyamines on the conformation of an oligodeoxyribonucleotide with the estrogen response element. *Nucleic Acids Res.* **25,** 2396–2402.
65. Manning, G. S. (1978) The molecular theory of polyelectrolyte solutions with applications to the electrostatic properties of polynucleotides. *Q. Rev. Biophys.* **11,** 179–246.
66. Bloomfield, V. A. (1997) DNA condensation by multivalent cations. *Biopolymers* **44,** 269–282.
67. Ouameur, A. A. and Tajmir-Riahi, H. A. (2004) Structural analysis of DNA interactions with biogenic polyamines and cobalt(III)hexamine studied by Fourier transform infrared and capillary electrophoresis. *J. Biol. Chem.* **279,** 42,041–42,054.
68. Ruiz-Chica, J., Medina, M. A., Sanchez-Jimenez, F., and Ramirez F. J. (2003) Raman spectroscopy study of the interaction between biogenic polyamines and an alternating AT oligodeoxyribonucleotide. *Biochim. Biophys. Acta* **1628,** 11–21.
69. Deng, H., Bloomfield, V. A., Benevides, J. M., and Thomas, G. J. Jr. (1999) Dependence of the Raman signature of genomic B-DNA on nucleotide base sequence. *Biopolymers* **50,** 656–666.
70. Thomas, T., Balabhadrapathruni, S., Gallo, M. A., and Thomas, T. J. (2002) Development of polyamine analogs as cancer therapeutic agents. *Oncol. Res.* **13,** 123–135.

7

Polyamine Metabolism and the Hypertrophic Heart

Lisa M. Shantz and Emanuele Giordano

1. Introduction

Cardiac muscle hypertrophy is one of the most important compensatory responses of the heart to multiple stresses that may be placed on it. If the stress is not relieved, sustained hypertrophy may progress to dysfunction and heart failure. The polyamines putrescine, spermidine, and spermine increase within hours of various types of experimentally induced cardiac hypertrophy, along with ornithine decarboxylase (ODC) gene expression. Several animal models have implicated ODC induction as an important factor in the development of hypertrophy, particularly in response to β-adrenergic stimulation. Novel transgenic mouse lines that overexpress several enzymes of polyamine metabolism in the heart have been generated in recent years, and crosses of these lines have pointed to decarboxylated adenosylmethionine and its control by *S*-adenosylmethionine decarboxylase as another important element in maintaining cardiac polyamine homeostasis. The activity of arginase is thought to play a regulatory role in the biosynthesis of both nitric oxide (NO) and polyamines, and NO deficiency has been linked to the development of cardiac hypertrophy. Genetically altered mouse lines with changes in arginine and NO metabolism in the heart are available, many of which possess cardiac abnormalities. These models will provide a valuable means to address the interdependence of arginine and ornithine metabolism in the development of myocardial hypertrophy and failure. Use of these tools may lead to a better understanding of the control of the signaling pathways that include the polyamines, arginine, and NO, allowing future work to focus on the interactions between these pathways in the development of heart disease.

2. Pathological Left Ventricular Hypertrophy

Cardiac hypertrophy and its transition to heart failure is a complex illness, and the most life-threatening disease in Western societies. Cardiac hypertrophy is defined as an increase in heart size mainly from an increased volume of muscle cells. Growth of the cardiac mass may be a physiological response, as observed in increased exercise,

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pregnancy, or childhood, or as part of a pathological response associated with hemodynamic overload, resulting in abnormal heart function. The hypertrophic response is initiated and sustained by a complex interplay of physical (mechanical stress) and neurohumoral (catecholamines, endothelin-1, angiotensin II) factors that in turn activate specific signaling cascades. Although the growth of the muscular mass is considered to be a compensatory mechanism maintaining systolic performance, myocardial hypertrophy has been shown to represent an independent risk factor for cardiovascular disease *(1)*.

Pathological left ventricular hypertrophy (LVH) changes the properties of the myocardial cells by decreasing contractile function, eventually leading to heart failure. As pathological LVH progresses, the fraction of cell volume occupied by the mass of myofilaments increases, causing cells to lose their plasticity. Synthetic activities of nonmuscles cells, such as fibroblasts, lead to myofibrillar disarray and fibrosis. Pathological LVH is characterized by increased production of contractile proteins and reactivation of a program of embryonic gene expression, including atrial natriuretic factor (ANF). ANF functions to reduce the load on the heart by increasing vasodilation and decreasing fluid accumulation (reviewed in ref. *2*).

Heart failure results when compensatory LVH progresses to dysfunction. Congestive heart failure can be caused by a number of alterations in the heart, including excessive pressure load from arterial hypertension, excessive volume overload, and hypertrophic or dilated cardiomyopathy *(2)*. The development of novel therapeutic strategies to prevent this progression from hypertrophy to failure depends on understanding pathological LVH at the molecular level.

The polyamines putrescine, spermidine, and spermine have been shown to be essential for cell growth and differentiation. It has been known for some time that intracellular polyamines increase within hours of various types of experimentally induced cardiac hypertrophy *(3)*. Most of this chapter will concentrate on the response of ODC, the first and potentially rate-limiting enzyme of polyamine biosynthesis, in animal models of the hypertrophic phenotype. Although the majority of the work on polyamine-metabolizing enzymes and heart hypertrophy has centered around ODC, transgenic mouse models with overexpression of *S*-adenosylmethionine decarboxylase (AdoMetDC) and spermine synthase in the heart also have been developed recently *(4)*. Extensive studies of hypertrophy in these models have yet to be completed. However, they will undoubtedly add to our knowledge of the regulation of polyamine metabolism in the hypertrophic phenotype in the near future. Additional information will be gained through the study of animals with altered regulation of the arginine-metabolizing enzymes, many of which have cardiac abnormalities. This chapter will also examine these models and their relevance to the study of polyamine metabolism in the heart.

3. Altered Polyamines in the Hypertrophic Phenotype

Initial observations pointing out the relationship between polyamines and cardiac hypertrophy date back more than 30 yr. In these studies, increases in polyamines were found in the ventricular myocardium of animals in which hypertrophy was induced by ascending aortic stenosis *(5)*, stress *(6)*, intense muscular exercise *(7)*, or the β-adrenergic

receptor agonist isoproterenol *(8)*. The rise in polyamine content was accompanied by increased histone acetylation *(9)*, and increased rates of both RNA synthesis *(7,9)* and protein synthesis *(10)*. Inhibition of polyamine synthesis decreased histone acetylation and RNA transcription, and this effect was reversed by spermine *(11)*. Later studies also implicated increased spermidine and spermine in hypertension and cardiac hypertrophy in a rat angiotensin II infusion model *(12)*.

A hallmark of the hypertrophic phenotype induced by either pressure overload or agonist administration is immediate early gene expression, including *c-fos*, *c-jun*, *c-myc*, and *ODC (2,13)*. *ODC* gene expression is accompanied by induction of ODC protein and activity. Increased ODC activity has been shown to occur quite rapidly in both mouse and rat models in response to various agents that induce a pathological cardiac hypertrophy, including thyroxine *(14)*, the β-adrenergic agonists isoproterenol (β1 and β2) and clenbuterol (β2) and the α_1-adrenergic agonists phenylephrine and methoxamine *(15–17)*. Another study has reported that ODC induction in response to isoproterenol depends upon increased transforming growth factor-β activity, both in primary myocyte culture and in isolated, perfused mouse hearts *(18)*.

Administration of α-difluoromethylornithine (DFMO), a suicide inactivator of ODC, reduced polyamine content and attenuated isoproterenol- and clenbuterol-induced cardiac hypertrophy *(15,16,18)*. Another inhibitor of ODC, α-methylornithine, also inhibited both ODC induction and development of a hypertrophic phenotype in cardiac myocytes with high transforming growth factor-β activity *(18)*. Interestingly, DFMO did not prevent thyroxine-induced hypertrophy *(13,14)*, and α-methylornithine did not attenuate phenylephrine-induced hypertrophy *(18)*. These results suggest ODC-dependent and ODC-independent processes leading to the hypertrophic state, depending on the inducer.

The induction of ODC activity in response to β-adrenergic agonists has been extensively studied in recent years. The β-adrenergic pathway is a critical point of hormonal control of cardiac contractility in both normal and failing hearts. Although the mechanism of induction of ODC during β-agonist-induced hypertrophy is still unknown, the promoter of the ODC gene contains a cyclic adenosine monophosphate (cAMP)-response element, and the β-adrenergic receptor pathway activated by agonists such as isoproterenol involves the stimulatory guanosine 5′-triphosphate regulatory protein (Gs), activation of adenylyl cyclase, and accumulation of cAMP *(19,20)*. ODC activity can also be induced in myocyte culture by dibutyryl-cAMP *(18)*.

Several studies have suggested that the induction of ODC in response to β-adrenergic stimulation is primarily through β2-adrenergic receptors, which are a minor component of the β-adrenergic receptors in the heart, but may become more important in the failing heart (reviewed in ref. *19*). Our studies using isoproterenol and the β1-receptor specific antagonist atenolol suggest that both β1 and β2 receptors contribute to the hypertrophy seen in the hearts of α-myosin heavy chain (MHC)-ODC transgenic mice, as discussed in the following section *(21)*. In addition, it has been shown that cardiac hypertrophy resulting from treatment with the β2-specific agonist clenbuterol results in an accumulation of polyamines in the heart, which can be reversed by DFMO. ODC induction by isoproterenol both in vivo and in primary myocyte culture could also be prevented by β2-receptor specific antagonists *(15,18,22)*.

4. Genetically Modified Mice

Transgenic and knockout mice represent powerful models for a deeper insight into the pathophysiological state, and several engineered strains have improved the knowledge of critical molecular steps responsible for development of the hypertrophic phenotype *(23)*. The similarities in cardiac hypertrophy and heart failure between mice and humans have shown that mice are a valid model for studying these disorders (reviewed in ref. *24*). Available mouse models with altered levels of polyamine-metabolizing enzymes in the heart, as well as models with alterations in the arginine-metabolizing enzymes (discussed later in this chapter) are listed in Table 1. These include transgenic mice in which ODC, AdoMetDC, spermidine synthase, or spermidine spermine N^1-acetyltransferase were increased generally in multiple tissues by use of their own promoters. This work has been reviewed elsewhere recently *(25)*.

Interestingly, with the exception of ODC overexpression that produces a moderate baseline hypertrophy, none of the transgenic lines expressing a single polyamine-metabolizing enzyme in the heart exhibit a cardiac phenotype. However, when these lines were crossed, several produced lethal phenotypes, particularly when one of the transgenes was AdoMetDC (Table 1). Homozygous deletion of either AdoMetDC *(26)* or ODC *(27)* also produced a lethal phenotype (Table 1). Deletion of the spermine synthase gene in a mouse line called Gy, which is the result of deletion of part of the X chromosome, produces a complex phenotype with several physical and behavioral alterations *(28)*. These mice are also prone to sudden death, suggesting the possibility of a cardiac abnormality such as arrhythmias, although these have not been reported (Table 1).

We have reported that the targeted expression of ODC to the heart enhances β-adrenergic agonist-induced cardiac hypertrophy *(21)*. Transgenic mice were generated with cardiac-specific expression of a stable ODC protein using the α-myosin heavy chain promoter (MHC-ODC mice). Founder lines with a 30-fold overaccumulation of putrescine, a fourfold elevation in spermidine, a slight increase in spermine, and accumulation of large amounts of cadaverine compared with littermate controls were established. Despite the alterations in polyamines in the transgenic heart, early studies detected no significant hypertrophy based on ratio of heart weight to body weight, although ANF mRNA was elevated in transgenic ventricles *(21)*. Subsequent studies after multiple backcrosses have revealed a moderate baseline hypertrophy, as measured by ratio of heart to body weight, myocyte cross-sectional area, and echocardiography *(29)*. Stimulation of β-adrenergic signaling by isoproterenol resulted in much more severe hypertrophy in MHC-ODC mice compared with littermates, without further alterations in the polyamines *(21,29)*. This murine genotype is also more prone than wild-type littermates to develop myocardial hypertrophy following pressure overload by aortic stenosis induced through abdominal aortic banding (E. Giordano and L. M. Shantz, unpublished observations).

Echocardiography of MHC-ODC mice revealed changes consistent with concentric hypertrophy, which is a common pathological response to pressure overload characterized by increased width of individual cardiac myocytes *(29)*. Despite the existing LVH,

Table 1
Genetically Engineered Mice With Cardiac Alterations in Polyamine-Metabolizing Enzymes or Arginine-Metabolizing Enzymes

Enzyme	Genetic manipulation	Target tissue	Baseline cardiac phenotype
Polyamine-metabolizing enzymes			
ODC	Genomic transgene (human ODC promoter)	Multiple	Phenotypically normal
	Transgene (αMHC promoter)	Heart	Moderate LV hypertrophy
	Knockout	Multiple	Lethal
AdoMetDC	Genomic transgene (rat AdoMetDC promoter)	Multiple	Phenotypically normal
	Transgene (αMHC promoter)	Heart	Phenotypically normal
	Knockout	Multiple	Lethal
AdoMetDC/ODC	Transgene/transgene (αMHC/αMHC)	Heart/heart	Lethal
SSAT	Genomic transgene (mouse SSAT promoter)	Multiple	Phenotypically normal
Antizyme-1	Transgene (αMHC promoter)	Heart	Phenotypically normal
Spermine synthase (SpmS)	Transgene (CAG promoter)	Multiple	Phenotypically normal
	Gene deletion (Gy mice)	Multiple	Sudden death
SpmS/ODC	Transgene/transgene (CAG/αMHC)	Multiple/ heart	Moderate LV hypertrophy
SpmS/ AdoMetDC	Transgene/transgene (CAG/αMHC)	Multiple/ heart	Lethal
Spermidine synthase	Genomic transgene (human spermidine synthase promoter)	Multiple	Phenotypically normal
Arginine-metabolizing enzymes			
Arginase I	Knockout	Multiple	Lethal (postnatal day 10–14)
Arginase II	Knockout	Multiple	Phenotypically normal
NOS1	Knockout	Multiple	Age-related LV hypertrophy
NOS2	Knockout	Multiple	No cardiac abnormalities
	Transgene (αMHC promoter)	Heart	No cardiac abnormalities
NOS3	Knockout	Multiple	Hypertension; decreased heart rate; age-related LV hypertrophy
	Genomic transgene (human NOS3 promoter)	Multiple	No cardiac abnormalities
	Transgene (preproendothelin-1 promoter)	Multiple	Hypotension
	Transgene (αMHC promoter)	Heart	Decreased LV pressure

significant changes in fractional shortening and ejection fraction were not detected in transgenic mice, suggesting that left ventricular function was well compensated *(29)*. However, staining of cardiac tissue with wheat germ agglutinin suggested an increase in interstitial collagen, which is indicative of fibrosis, in the hearts of MHC-ODC mice, both in the basal state and even higher after treatment with isoproterenol *(29)*.

It is unknown why the high ODC levels in the hearts of MHC-ODC mice leads to the magnified response to isoproterenol. However, there is evidence for the involvement of apoptotic pathways during the transition between cardiac hypertrophy and heart failure in animal models (reviewed in ref. *23*), and several in vitro studies have indicated that loss of ODC regulation and overaccumulation of putrescine result in apoptosis *(30,31)*. These studies raise the possibility that ODC-overexpressing hearts are more susceptible to induction of apoptosis in response to stress than normal hearts because of their extremely high levels of putrescine. However, other studies have shown that polyamine accumulation leads to a protective effect in the presence of apoptotic stimuli, and depletion induces apoptosis (reviewed in ref. *32*).

Although the ODC levels in the hearts of MHC-ODC mice are substantially increased when assayed in vitro, it is likely that the resulting increase in polyamines in the heart is limited by the availability of L-ornithine and of decarboxylated *S*-adenosylmethionine (dcAdoMet). It has been shown in several in vitro systems that high levels of putrescine do not always lead to an increase in the higher polyamines *(33,34)*, pointing to AdoMetDC and not ODC as the rate-limiting factor in spermidine and spermine synthesis. Indeed, despite more than a 1000-fold overexpression of ODC and 30-fold enlargement of putrescine pools reported in MHC-ODC mice, spermidine was elevated only fourfold and spermine less than twofold *(21)*. Although this represents a more substantial increase in spermidine compared with other systems with ODC overaccumulation, the inability to increase tissue spermine levels in the presence of high ODC activity has been noted in other transgenic models (reviewed in ref. *25*). In addition to limited availability of dcAdoMet, another explanation for this phenomenon could be sequestration of putrescine pools, such that putrescine becomes unavailable for further metabolism to the higher polyamines.

Mice with constitutive overexpression of AdoMetDC in the heart have been generated recently using the cardiac-specific αMHC promoter and found to be phenotypically normal *(4)*. Crosses in which both AdoMetDC and ODC are expressed at high levels in the heart are lethal *in utero* (Neisenberg, O., Shantz, L. M., and Pegg, A. E., unpublished observation). Similarly, when MHC-AdoMetDC mice were crossed with CAG/SpmS mice, which have a general overexpression of spermine synthase in many organs, including the heart, the combination was lethal at an early age *(4)*. Although the cause of this lethality is not known, it is possible that the overexpression of AdoMetDC is able to shift all of the higher polyamines in the heart toward the formation of spermine because of the presence of excess dcAdoMet and high levels of spermine synthase. It has been suggested that spermine is the most effective of the polyamines in altering inward rectification of potassium channels and in inducing apoptosis *(35,36)*. Thus high levels of spermine may cause lethal effects by disturbing the normal functions of these pathways in the heart. It should be noted that CAG/SpmS mice, despite up to 1000-fold induction

of spermine synthase in multiple organs, including the heart, are phenotypically normal with only a modest rise in cardiac spermine levels. This agrees with the idea of dcAdoMet levels being limiting unless AdoMetDC is also overexpressed.

Breeding of MHC-ODC mice with CAG-SpmS mice produced offspring with more than 1000-fold induction of both ODC and spermine synthase in the heart. This profound change in two polyamine metabolizing enzymes produced a 44% decrease in cardiac spermidine (a reduction almost to nontransgenic control levels) and only a 20% increase in cardiac spermine levels compared with MHC-ODC mice, again pointing to the importance of dcAdoMet in polyamine homeostasis *(4)*. CAG-SpmS/MHC-ODC mice maintain the moderate LVH seen in MHC-ODC mice *(4)*, suggesting the increased spermidine in MHC-ODC mice is not related to the observed hypertrophy.

Definitive proof that high levels of ODC activity are necessary to induce a phenotype of hypertrophy or failure in vivo would ideally require gene deletion studies. In the case of *ODC*, which is a housekeeping gene essential for normal growth and development, homozygous deletion has been shown to be lethal very early in embryonic development *(27)*. As an alternative approach to address this issue, a transgenic mouse line expressing the protein antizyme (ATZ) under the control of the αMHC promoter was generated *(37)*. In addition to promoting the degradation of ODC, binding of ATZ also inhibits ODC activity, and overexpression of ATZ can inhibit polyamine transport into the cell, making it difficult for the cell to compensate for reduced ODC activity by importing polyamines *(38)*. This points to an advantage of ATZ-overexpressing mice over both ODC knockout mice and treatment of normal mice with DFMO. In addition, mice pretreated with DFMO are much less responsive to isoproterenol than mice that received DFMO and isoproterenol simultaneously *(13)*, suggesting prevention of the initial rise in ODC activity is more effective in reducing the occurrence of subsequent hypertrophy.

Overexpression of ATZ driven by the αMHC promoter (MHC-ATZ mice) caused no abnormal phenotype and very little change in polyamines in the hearts of untreated mice. This observation can be explained by the continued presence of ODC activity in the hearts of MHC-ATZ mice *(37)*. When MHC-ATZ mice were treated with isoproterenol, ODC activity in the heart was depressed compared with control mice, but two different lines of MHC-ATZ mice exhibited the same hypertrophic response as their littermates *(37)*. These results suggest that β-agonist-induced hypertrophy is the result of the concurrent activation of multiple, possibly independent, signaling pathways. Only one marker of hypertrophy was evaluated in these studies (ratio of heart weight to body weight), and it is not known whether other genetic or biochemical markers of hypertrophy were changed in MHC-ATZ mice. In addition, one of the MHC-ATZ lines showed a slight hypertrophic phenotype in untreated mice. It is unknown whether the high expression of ATZ in the hearts of these mice might affect other pathways involved in the development of hypertrophy. Another question remaining to be answered is the nature of the residual ODC activity in MHC-ATZ hearts mentioned earlier in this chapter. Compartmentalization of some fraction of the ODC protein might allow a local induction in response to the hypertrophic stimulus even in the presence of very high levels of ATZ.

5. Role of Arginine-Metabolizing Enzymes

The majority of work completed to date on ODC and cardiac hypertrophy has measured the effects of altered ODC activity only on the polyamines, which are downstream products of the ODC reaction. However, the ODC protein is part of a more complex signaling cascade (Fig. 1). The ODC substrate ornithine is produced in all cells by the activity of arginase, which catalyzes the conversion of L-arginine to L-ornithine and urea *(39)*. Arginase exists as two isoforms, with arginase I targeted to the cytoplasm and arginase II to the mitochondria *(39)*. ODC is extramitochondrial, whereas ornithine may be located in the mitochondria or cytoplasm (Fig. 1). L-arginine is also the substrate for NO synthases (NOS). All three NOS isoforms catalyze the synthesis of NO and L-citrulline from L-arginine *(40)*. Thus the conversion of L-arginine into L-ornithine by arginase limits the availability of L-arginine as a precursor of NO and shifts the equilibrium between NOS and ODC toward polyamine biosynthesis *(41)*. The activity of arginase is therefore thought to play a regulatory role in the biosynthesis of both NO and polyamines *(41)*.

NO production is involved in a large number of physiological and pathophysiological functions in animal tissues, and the cardiovascular system is dramatically affected by its availability. Among its actions, NO is responsible for endothelium-mediated vasorelaxing tone, prevents platelet aggregation and adhesion to the vessel wall, and is the mediator of parasympathetic neurovegetative tone in the myocardium. In addition, several reports have pointed out the antiproliferative effect of NO in smooth muscle cells in the pathogenesis of atherosclerosis *(42)* and the antihypertrophic potential of NO in the heart *(43)*.

In agreement with their opposing effects on cell proliferation, polyamines can inhibit NOS activity and NO-mediated effects *(44,45)*. In addition, NO and its intermediate NG-hydroxy-L-arginine have been identified as inhibitors of ODC and polyamine-mediated pathways *(46)*. Other studies have shown that NG-hydroxy-L-arginine inhibits arginase activity *(47)* and limits cell proliferation *(48)* in several biological systems.

As with ODC induction, NO deficiency has also been linked to the development of cardiac hypertrophy using mouse models, as shown in Table 1 *(43)*. NO has been shown to diminish the β-adrenergic response in cardiomyocytes *(49)*, and overexpression of NOS 3 in the heart using the preproendothelin-1 promoter was found to attenuate cardiac hypertrophy in response to isoproterenol, as measured by the decreased ratio of heart weight to body weight and myocyte cross-sectional area *(50)*. ANF-upregulation in response to isoproterenol was also inhibited in NOS 3 transgenic mice. Another transgenic NOS 3 line that made use of the native NOS 3 promoter to produce moderate elevations of protein and preserve transcriptional regulation showed a dramatic increase in survival with reduction of pulmonary edema and improved cardiac function in NOS 3-overexpressing mice subjected to infarct-induced congestive heart failure *(51)*.

The findings described support strongly the differential regulation of NO and polyamine biosynthesis through the availability of arginine. Consistent with this idea is the observation that when cardiac hypertrophy was induced in MHC-ODC mice with isoproterenol, arginase activity was induced dramatically, whereas activity in littermate controls was below the limit of detection *(29)*. The transgenic phenotype is also characterized by a twofold upregulation of NOS 3 protein in the heart *(29)*. Changes in

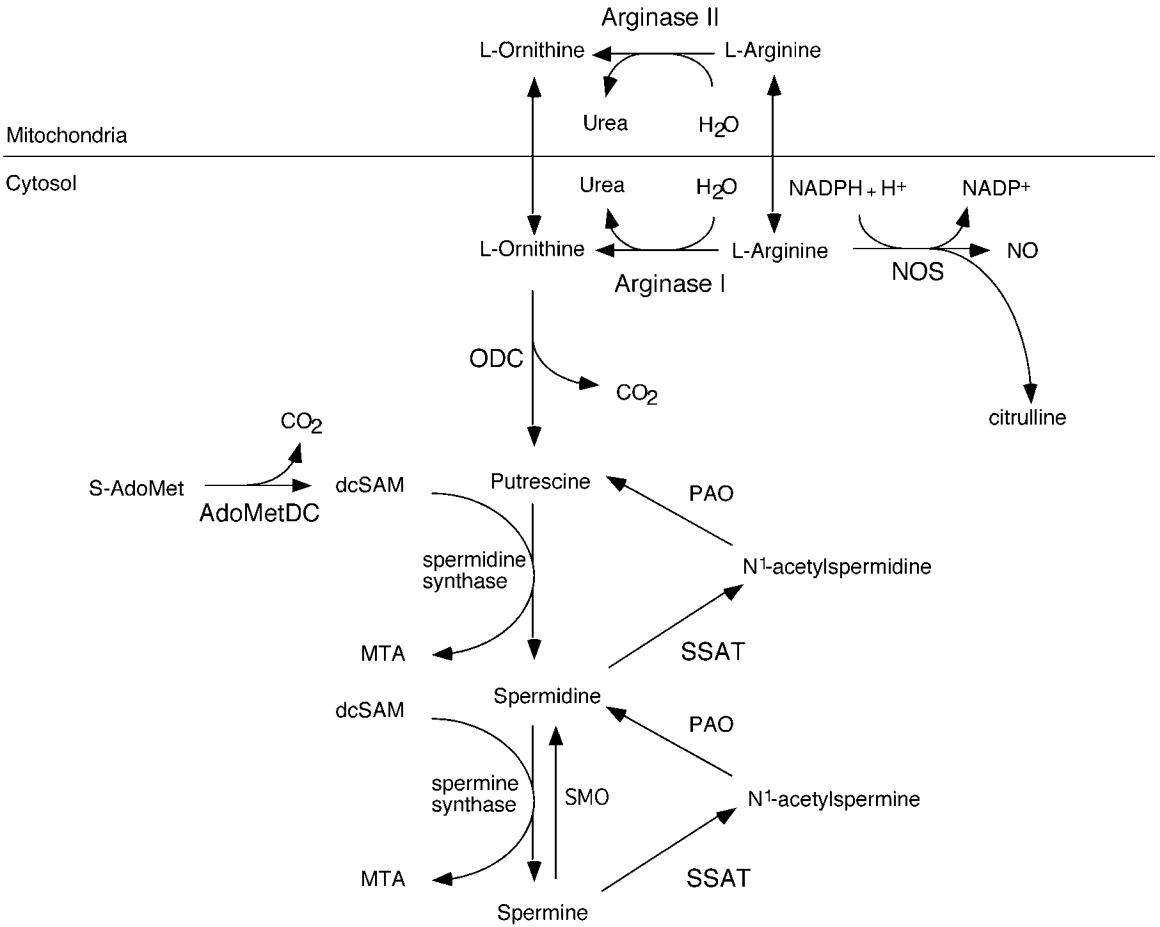

Fig. 1. The polyamine and arginine metabolic pathways.

L-arginine availability may therefore contribute to differential regulation of NO and polyamine biosynthesis in MHC-ODC mice. Little is known about the levels of the polyamines or their enzymes in the hearts of NOS knockout mice or mice with cardiac-specific NOS overexpression. However, the results obtained with MHC-ODC mice indicate that these questions are worth pursuing.

The overproduction of polyamines, induction of arginine-metabolizing enzymes, and activation of β-adrenergic pathways may interact through several possible mechanisms. For example, activation of β-adrenergic receptors causes Ca^{2+} mobilization through phosphorylation of the L-type calcium channel by the cAMP-dependent protein kinase A, and also through direct coupling of stimulatory guanosine 5′-triphosphate regulatory protein to calcium channel activation *(19)*. It has been suggested that polyamines may play a role in stimulation of Ca^{2+} influx in rat myocytes in the presence of isoproterenol *(52)*. Thus the positively charged polyamines, particularly the diamine putrescine, may affect Ca^{2+} mobilization in response to β-adrenergic receptor activation. It is also known that NO inhibits L-type Ca^{2+} channels *(53)*, and it has been shown that NOS 3 localizes to caveolae, where it is compartmentalized with β-adrenergic receptors and L-type Ca^{2+} channels *(54)*. Therefore, the increase in NOS 3 in the hearts of MHC-ODC mice may represent an attempt to supply an adaptive counterresponse to the hypertrophic stimulus. This is also suggested by evidence in the literature pointing to the ability of NO to downregulate ODC activity *(46)* and to blunt the hypertrophic potential of a variety of myocardial stressors *(43)*.

6. Future Directions

The ability to maintain polyamine homeostasis in the heart despite substantial, simultaneous induction of ODC and spermine synthase points to dcAdoMet and its control by AdoMetDC as an important area of future study. The MHC-AdoMetDC mice have no baseline hypertrophy, however, crosses of these mice with others possessing altered polyamine regulation have proven to be lethal. Studies of these mice with inducers of a hypertrophic phenotype have not yet been reported. However, use of newer transgenic technology, such as inducible promoters and conditional knockouts, may be necessary to address the role of spermine and the ratio of spermine to spermidine in the development of the hypertrophic phenotype.

Although many studies have established that increases in polyamines are an early event in the development of cardiac hypertrophy, little is known of the regulation of polyamine levels in the progression from hypertrophy to heart failure. Mice have also been used as a model for congestive heart failure by induction of myocardial infarction using descending coronary artery occlusion, in which animals may be monitored for an extended period after infarction (*see* ref. *51*). This model may be useful in studying polyamine regulation in both transgenic and nontransgenic mice. Of particular interest would be the potential protective effect of ATZ overexpression in this model, perhaps implicating ODC as a target of therapy for patients with congestive heart failure.

There are a variety of genetically altered mouse strains available that are promising candidates for cross-breeding with mice that have altered cardiac polyamines. These include mice deficient in all three isoforms of NOS *(54)*, transgenic NOS mice

(50,51,55,56), and mice containing a targeted disruption of the arginase II gene *(57)* (*see* Table 1). These models can be used to increase understanding of the association between polyamine regulation and arginine regulation in both the normal and hypertrophic heart. Arginase II ($^{-/-}$) mice showed a trend toward increased plasma ornithine, although the difference was not statistically significant *(57)*. A homozygous arginase I deletion has been shown to be lethal early in life *(58)*. However, arginase I ($^{+/-}$) mice are viable, and may also be useful, particularly in crossing with mice containing altered ODC in the heart. The oral supplementation of L-arginine has been suggested as a tool to increase the activity of the NO/NOS biochemical pathway. This intervention could also be relevant in understanding the mechanism of the hypertrophic phenotype obtained in ODC-overexpressing mice.

In addressing the relationship between polyamine regulation and β-adrenergic activation, transgenic lines overexpressing both β1- and β2-adrenergic receptors under control of the αMHC promoter have been described *(59,60)*, as well as mice overexpressing proteins downstream of these receptors, such as the Gαs protein *(61)* and adenylyl cyclase *(62)*. These models can be used to address whether pathways downstream of the β-adrenergic receptors are directly connected to alterations in the polyamines, or act in parallel with increased polyamines in the development of a pathological cardiac phenotype.

Several of the lines mentioned here develop age-related hypertrophy (e.g., NOS1($^{-/-}$) mice, NOS3($^{-/-}$) mice, αMHC-β2-AR mice). Because hypertrophy and its transition to heart failure is often a disease of aging, these models are physiologically relevant. Breeding of these mice with MHC-ATZ mice would address the importance of ODC induction in long-term studies, rather than the more acute phenotype generated by β-adrenergic agonist stimulation. Other available models show improved function in cardiomyopathy (αMHC-AC mice), making them an interesting choice for breeding not only with MHC-ODC mice, but also with either CAG-SpmS mice or MHC-AdoMetDC mice. Experiments such as these would address the importance of spermine itself, as well as the ratio of spermidine to spermine in the diseased heart. Thus these examples provide interesting possibilities for creating strains that have modifications in a variety of signal transduction pathways along with deregulated polyamine metabolism, providing a powerful technique to determine important interactions that mediate or antagonize the effects of altered polyamine levels in the heart.

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References

1. Levy, D., Garrison, R. J., Savage, D. D., Kannel, W. B., and Castelli, W. P. (1990) Prognostic implications of echocardiographically determined left ventricle mass in the Framingham heart study. *N. Engl. J. Med.* **322,** 1561–1566.

2. Sugden, P. H. and Clerk, A. (1998) Cellular mechanisms of cardiac hypertrophy. *J. Mol. Med.* **76,** 725–746.
3. Flamigni, F., Rossoni, C., Stefanelli, C., and Caldarera, C. M. (1986) Polyamine metabolism and function in the heart. *J. Mol. Cell. Cardiol.* **18,** 3–11.
4. Ikeguchi, Y., Wang, X., McCloskey, D. E., et al. (2004) Characterization of transgenic mice with widespread overexpression of spermine synthase. *Biochem. J.* **381,** 701–707.
5. Caldarera, C. M., Casti, A., Rossoni, C., and Visioli, O. (1971) Polyamines and noradrenaline following myocardial hypertrophy. *J. Mol. Cell. Cardiol.* **3,** 121–126.
6. Russell, D. H., Shiverick, K. T., Hamrell, B. B., and Alpert, N. R. (1971) Polyamine synthesis during initial phases of stress-induced cardiac hypertrophy. *Am. J. Physiol.* **221,** 1287–1291.
7. Caldarera, C. M., Orlandini, G., Casti, A., and Moruzzi, G. (1974) Polyamines and nucleic acid metabolism in myocardial hypertrophy of the overloaded heart. *J. Mol. Cell. Cardiol.* **6,** 95–104.
8. Warnica, J. W., Antony, P., Gibson, K., and Harris, P. (1975) The effect of isoprenaline and propranolol on rat myocardial ornithine decarboxylase. *Cardiovasc. Res.* **9,** 793–796.
9. Moruzzi, G., Caldarera, C. M., and Casti, A. (1974) The biological effect of polyamines on heart RNA and histone metabolism. *Mol. Cell. Biochem.* **3,** 153–161.
10. Gibson, K. and Harris, P. (1974) The in vitro and in vivo effects of polyamines on cardiac protein biosynthesis. *Cardiovasc. Res.* **8,** 668–673.
11. Caldarera, C. M., Casti, A., Guarnieri, C., and Moruzzi, G. (1975) Regulation of ribonucleic acid synthesis by polyamines. *Biochem. J.* **152,** 91–98.
12. Ibrahim, J., Schachter, M., Hughes, A. D., and Sever, P. S. (1995) Role of polyamines in hypertension induced by angiotensin II. *Cardiovasc. Res.* **29,** 50–56.
13. Bartolome, J., Huguenard, J., and Slotkin, T. A. (1980) Role of ornithine decarboxylase in cardiac growth and hypertrophy. *Science* **210,** 793–794.
14. Pegg, A. E. (1981) Effect of α-difluoromethylornithine on cardiac polyamine content and hypertrophy. *J. Mol. Cell. Cardiol.* **13,** 881–887.
15. Cubria, J. C., Reguera, R., Balana-Fouce, R., Ordonez, C., and Ordonez, D. (1998) Polyamine-mediated heart hypertrophy induced by clenbuterol in the mouse. *J. Pharm. Pharmacol.* **50,** 91–96.
16. Tipnis, U. R., He, G. Y., Campbell, G., and Boor, P. J. (2000) Attenuation of isoproterenol-mediated myocardial injury in rat by an inhibitor of polyamine synthesis. *Cardiovasc. Pathol.* **9,** 273–80.
17. Thompson, K. E., Friberg, P., and Adams, M. A. (1992) Vasodilators inhibit acute alpha 1-adrenergic receptor-induced trophic responses in the vasculature. *Hypertension* **20,** 809–815.
18. Schluter, K. D., Frischfopf, K., Flesch, M., Rosenkranz, S., Taimor, G., and Piper, H. M. (2000) Central role for ornithine decarboxylase in β-adrenoceptor mediated hypertrophy. *Cardiovasc. Res.* **45,** 410–417.
19. Steinberg, S. F. (1999) The molecular basis for distinct β-adrenergic receptor subtype actions in cardiomyocytes. *Circ. Res.* **85,** 1101–1111.
20. Palvimo, J. J., Eisenberg, L. M., and Jänne, O. A. (1991) Protein-DNA interactions in the cAMP responsive promoter region of the murine ornithine decarboxylase gene. *Nucleic Acids Res.* **19,** 3921–3927.
21. Shantz, L. M., Feith, D. J., and Pegg, A. E. (2001) Targeted overexpression of ornithine decarboxylase enhances β-adrenergic agonist-induced cardiac hypertrophy. *Biochem. J.* **358,** 25–32.

22. Nakano, M., Kanada, T., Matsuzaki, S., et al. (1995) Effect of losartan, an AT_1 selective angiotensin II receptor antagonist, on isoproterenol-induced cardiac ornithine decarboxylase activity. *Res. Commun. Mol. Pathol. Pharmacol.* **88,** 21–30.
23. Hunter, J. J. and Chien, K. R. (1999) Signaling pathways for cardiac hypertrophy and failure. *N. Engl. J. Med.* **341,** 1276–1283.
24. Chu, G., Haghighi, K., and Kranias, E. G. (2002) From mouse to man: understanding heart failure through genetically altered mouse models. *J. Card. Failure* **8,** S432–S449.
25. Janne, J., Alhonen, L., Pietila, M., and Keinanen, T. A. (2004) Genetic approaches to the cellular functions of polyamines in mammals. *Eur. J. Biochem.* **271,** 877–894.
26. Nishimura, K., Nakatsu, F., Kashiwagi, K., Ohno, H., Saito, T., and Igarashi, K. (2002) Essential role of S-adenosylmethionine decarboxylase in mouse embryonic development. *Genes Cells* **7,** 41–47.
27. Pendeville, H., Carpino, N., Marine, J. C., et al. (2001) The ornithine decarboxylase gene is essential for cell survival during early murine development. *Mol. Cell. Biol.* **21,** 6549–6558.
28. Lorenz, B., Francis, F., Gempel, K., et al. (1998) Spermine deficiency in Gy mice caused by deletion of the spermine synthase gene. *Hum. Mol. Genet.* **7,** 541–547.
29. Hillary, R. A., Giordano, E., Pegg, A. E., et al. A lethal phenotype in mice with cardiac overexpression of ornithine decarboxylase. *Cardiovasc. Res.* In press.
30. Xie, X., Tome, M. E., and Gerner, E. W. (1997) Loss of intracellular putrescine pool-size regulation induces apoptosis. *Exp. Cell. Res*. **230,** 386–392.
31. Tome, M. E., Fiser, S. M., Payne, C. M., and Gerner, E. W. (1997) Excess putrescine inhibits the formation of modified eukaryotic initiation factor 5A (eIF-5A) and induces apoptosis. *Biochem. J.* **328,** 847–854.
32. Schipper, R. G., Penning, L. C., and Verhofstad, A. A. (2000) Involvement of polyamines in apoptosis. Facts and controversies: effectors or protectors? *Semin. Cancer Biol.* **10,** 55–68.
33. Shantz, L. M., Hu, R.-H., and Pegg, A. E. (1996) Regulation of ornithine decarboxylase in a transformed cell line that overexpresses translation initiation factor eIF-4E. *Cancer Res.* **56,** 3265–3269.
34. Tobias, K. E. and Kahana, C. (1995) Exposure to ornithine results in excessive accumulation of putrescine and apoptotic cell death in ornithine decarboxylase overproducing mouse myeloma cells. *Cell Growth Differentiation* **6,** 1279–1285.
35. Stefanelli, C., Stanic, I., Zini, M., et al. (2000) Polyamines directly induce release of cytochrome *c* from heart mitochondria. *Biochem. J.* **347,** 875–880.
36. Phillips, L. R. and Nichols, C. G. (2003) Ligand-induced closure of inward rectifier Kir6.2 channels traps spermine in the pore. *J. Gen. Physiol.* **122,** 795–804.
37. Mackintosh, C. A., Feith, D. J., Shantz, L. M., and Pegg, A. E. (2000) Overexpression of antizyme in the hearts of transgenic mice prevents the isoprenaline-induced increase in cardiac ornithine decarboxylase activity and polyamines, but does not prevent cardiac hypertrophy. *Biochem. J.* **350,** 645–653.
38. Hayashi, S. and Murakami, Y. (1995) Rapid and regulated degradation of ornithine decarboxylase. *Biochem. J.* **306,** 1–10.
39. Wu, G. and Morris, S. M., Jr. (1998) Arginine metabolism: nitric oxide and beyond. *Biochem. J.* **336,** 1–17.
40. Palmer, R. M., Ashton, D. S., and Moncada, S. (1988) Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* **333,** 664–666.
41. Li, H., Meininger, C. J., Hawker, J. R., Jr., et al. (2001) Regulatory role of arginase I and II in nitric oxide, polyamine, and proline syntheses in endothelial cells. *Am. J. Physiol. Endocrinol. Metab.* **280,** E75–E82.

42. Ignarro, L. J., Buga, G. M., Wei, L. H., Bauer, P. M., Wu, G., and del Soldato, P. (2001) Role of the arginine-nitric oxide pathway in the regulation of vascular smooth muscle cell proliferation. *Proc. Natl. Acad. Sci. USA* **98,** 4202–4208.
43. Simko, F. and Simko, J. (2000) The potential role of nitric oxide in the hypertrophic growth of the left ventricle. *Physiol. Res.* **49,** 37–46.
44. Hu, J., Mahmoud, M. I., and El-Fakahany, E. E. (1994) Polyamines inhibit nitric oxide synthase in rat cerebellum. *Neurosci. Lett.* **175,** 41–45.
45. Blachier, F., Selamnia, M., Robert, V., M'Rabet-Touil, H., and Duée, P. H. (1995) Metabolism of L-arginine through polyamine and nitric oxide synthase pathways in proliferative or differentiated human colon carcinoma cells. *Biochim. Biophys. Acta*. **1268,** 255–262.
46. Bauer, P. M., Buga, G. M., Fukuto, J. M., Pegg, A. E., and Ignarro, L. J. (2001) Nitric oxide inhibits ornithine decarboxylase via S-nitrosylation of cysteine 360 in the active site of the enzyme. *J. Biol. Chem.* **276,** 34,458–34,464.
47. Daghigh, F., Fukuto, J. M., and Ash, D. E. (1994) Inhibition of rat liver arginase by an intermediate in NO biosynthesis, NG-hydroxy-L-arginine: implications for the regulation of nitric oxide biosynthesis by arginase. *Biochem. Biophys. Res. Commun.* **202,** 174–180.
48. Buga, G. M., Wei, L. H., Bauer, P. M., Fukuto, J. M., and Ignarro, L. J. (1998) NG-hydroxy-L-arginine and nitric oxide inhibit Caco-2 tumor cell proliferation by distinct mechanisms. *Am. J. Physiol.* **275,** R1256–R1264.
49. Balligand, J.-L. (1999) Regulation of cardiac β-adrenergic response by nitric oxide. *Cardiovasc. Res.* **43,** 607–620.
50. Ozaki, M., Kawashima, S., Yamashita, T., et al. (2002) Overexpression of endothelial nitric oxide synthase attenuates cardiac hypertrophy induced by chronic isoproterenol infusion. *Circ. J.* **66,** 851–856.
51. Jones, S. P., Greer, J. M., van Haperen, R., Duncker, D. J., de Crom, R., and Lefer, D. J. (2003) Endothelial nitric oxide synthase overexpression attenuates congestive heart failure in mice. *Proc. Natl. Acad. Sci. USA* **100,** 4891–4896.
52. Fan, C. C. and Koenig, H. (1988) The role of polyamines in β-adrenergic stimulation of calcium influx and membrane transport in rat heart. *J. Mol. Cell. Cardiol.* **20,** 789–799.
53. Merry, P. F., Pavoine, C., Belhassen, L., Pecker, F., and Fischmeister, R. (1993) Nitric oxide regulates Ca^{2+} current. Involvement of cGMP-inhibited and cGMP-stimulated phosphodiesterases through guanylyl cyclase activity. *J. Biol. Chem.* **268,** 26,286–26,295.
54. Barouch, L. A., Harrison, R. W., Skaf, M. W., et al. (2002) Nitric oxide regulates the heart by spatial confinement of nitric oxide synthase isoforms. *Nature* **416,** 337–340.
55. Brunner, F., Andrew, P., Wolkart, G., Zechner, R., and Mayer, B. (2001) Myocardial contractile function and heart rate in mice with myocyte-specific overexpression of endothelial nitric oxide synthase. *Circulation* **104,** 3097–3102.
56. Heger, J., Godecke, A., Flogel, U., et al. (2002) Cardiac-specific overexpresseion of inducible nitric oxide synthase does not result in severe cardiac dysfunction. *Circ. Res.* **90,** 93–99.
57. Shi, O., Morris, S. M., Jr., Zoghbi, H., Porter, C. W., and O'Brien, W. E. (2001) Generation of a mouse model for arginase II deficiency by targeted disruption of the arginase II gene. *Mol. Cell. Biol.* **21,** 811–813.
58. Iyer, R. K., Yoo, P. K., Kern, R. M., et al. (2002) Mouse model for human arginase deficiency. *Mol. Cell. Biol.* **22,** 4491–4498.
59. Engelhardt, S., Hein, L., Wiesmann, F., and Lohse, M. J. (1999) Progressive hypertrophy and heart failure in β1-adrenergic receptor transgenic mice. *Proc. Natl. Acad. Sci. USA* **96,** 7059–7064.

60. Du, X.-J., Gao, X.-M., Wang, B., Jennings, G. L., Woodcock, E. A., and Dart, A. M. (2000) Age-dependent cardiomyopathy and heart failure phenotype in mice overexpressing β_2-adrenergic receptors in the heart. *Cardiovasc. Res.* **48,** 448–454.
61. Iwase, M., Bishop, S. P., Uechi, M., et al. (1996) Adverse effects of chronic endogenous sympathetic drive induced by cardiac GS alpha overexpression. *Circ. Res.* **78,** 517–524.
62. Roth, D. M., Bayat, H., Drumm, J. D., et al. (2002) Adenylyl cyclase increases survival in cardiomyopathy. *Circulation* **105,** 1989–1994.

8

Influence of Polyamines on Breast Cancer Biology

Andrea Manni

1. Introduction

More than 200,000 women are diagnosed yearly with breast cancer in the United States. Despite the significant improvement in survival introduced by both adjuvant hormonal therapy (restricted to estrogen receptor-positive tumors) and chemotherapy, approx 40,000 American women still die yearly from this malignancy. Therefore, improved understanding of the steps involved in carcinogenesis, cancer cell proliferation, and tumor progression is of critical importance to develop specifically targeted preventive and therapeutic regimens. The appreciation of the major role played by estrogens in human breast cancer biology has led to the development of effective treatments aimed at blocking either estrogen action with antiestrogens *(1)* or estrogen biosynthesis with aromatase inhibitors *(2)*. Both classes of compounds represent classical examples of biologically based therapies and have been found to be highly effective in the treatment of advanced disease, in the adjuvant setting and, at least with antiestrogens, in breast cancer prevention *(1,2)*. Unfortunately, these compounds are ineffective in the treatment of tumors that are either already hormone independent at diagnosis (approximately two-thirds of breast cancers) or which have progressed to hormone independence after an initial period of hormone responsiveness. Therefore, considerable effort is being placed in targeting multiple growth factor and other oncogenic signaling pathways whose activation may be responsible for the development of hormone resistance. It is hoped that these treatments may either prevent progression to hormone independence or may induce regression of tumors that have already become hormone resistant.

Over the years, our laboratory has been interested in exploring the polyamine pathway as a therapeutic target in breast cancer. Our interest in this approach stems from the evidence (to be reviewed here) that polyamines are involved in the hormonal control of breast cancer growth, and play a critical role in the expression of the invasive and metastatic properties of hormone-independent tumors. Activation of the polyamine metabolic pathway, typically leading to accumulation of intracellular polyamines, plays

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a major role in cancer biology, including breast cancer *(3,4)*. Most of the research has focused on ornithine decarboxylase (ODC), the first and rate-limiting enzyme in polyamine biosynthesis. Elevated levels of ODC activity have been demonstrated in a large variety of cancers compared with the surrounding normal tissues. Less is known about the role played in cancer by *S*-adenosylmethionine decarboxylase (AdoMetDC), the other highly inducible polyamine biosynthetic enzyme that promotes the formation of spermidine and spermine. We will provide a brief overview on the influence of polyamines on mammary carcinogenesis, breast cancer cell proliferation, and tumor progression, with particular focus on the development of metastasis. We will also discuss the possible cellular mechanisms mediating polyamine effects on breast cancer phenotype, mostly focusing on ongoing research in our laboratory. In the aggregate, the data point to the polyamine pathway as an attractive target for breast cancer prevention and treatment.

2. Mammary Carcinogenesis

Considerable evidence indicates that increased ODC activity and cellular polyamine levels are involved in carcinogenesis. Increased ODC activity is frequently detected in transformed cell lines *(5)*, animal tumors *(6)*, and some preneoplastic lesions *(7)*. In addition, activation of the polyamine pathway has been found to be essential in several experimental systems of carcinogenesis *(8,9)*. The observation that levels of polyamines and ODC activity are higher in human breast cancer specimens than in normal and benign breast tissue *(10,11)* provides support for a role of the polyamine pathway in breast cancer development. Using an immunohistochemical method that can be applied to formalin-fixed, paraffin-embedded tissue sections, we have found that ODC expression is significantly higher in the malignant epithelial component than in normal-appearing epithelial cells and stroma admixed with the tumor *(12)*, thus supporting the concept that ODC is upregulated during mammary carcinogenesis.

To test directly the role of ODC in mammary carcinogenesis, we induced overexpression of this enzyme in the spontaneously immortalized MCF-10A human mammary epithelial cell line *(13)*. We observed that induction of ODC overexpression markedly potentiated the transforming effect of HER-2neu and epidermal growth factor (EGF) receptor activation in our system *(13)*. Our data also suggested that activation of the mitogen-activated protein kinase (MAPK) cascade may mediate the cooperativity between the polyamine pathway and HER-2neu/EGF signaling in promoting clonogenicity (as an indicator of transformation) of MCF-10A cells *(13)*. Of note, in the absence of EGF and HER-2neu activation, ODC overexpression failed to induce both clonogenicity and MAPK activation *(13)*. This observation is in agreement with several lines of evidence indicating that ODC overexpression alone is not sufficient for the acquisition of a fully transformed phenotype *(14)*. The significance of the observed activation of the MAPK pathway in the presence of ODC overexpression and enhanced HER-2neu/EGF signaling is strengthened by the report of Sivaraman et al. *(15)*. These investigators detected a 5- to 10-fold increase in MAPK activity and expression in human breast cancer specimens over benign conditions, thus implicating this signal transduction pathway in breast cancer development. In studies performed in

murine fibroblast, Kubota et al. *(16)* observed enhanced MAPK activity in transformed ODC-overproducing transfectants. Therefore, ODC-induced activation of MAPK signaling is not restricted to our experimental system, but more likely is a central mediator of ODC-mediated transformation.

Additional evidence implicating polyamines in breast cancer development is provided by the finding that inhibition of ODC activity with α-difluoromethylornithine (DFMO) inhibits the development of chemically induced rat mammary tumors *(17)*. This protection is associated with suppressed ODC activity and polyamine levels, and is blocked by the addition of putrescine to the diet *(17)*. DFMO administration has also been shown to suppress the development of hormone-independent breast cancers in a transgenic model system *(18)*. This is a particularly important observation because, at the moment, there are no known regimens able to reduce the incidence of hormone-independent breast cancers, which are the most aggressive and whose development is not inhibited by antiestrogen treatment with tamoxifen.

Based on the recognized importance of ODC in carcinogenesis, DFMO treatment is being tested in several phase I and II chemopreventive trials *(19)*. Of particular relevance to breast cancer is a double-blind, randomized, phase II study conducted in a group of women at high risk *(20)*. The subjects were required to have pretreatment periareolar fine-needle aspiration cytology showing hyperplasia or hyperplasia with atypia. In this study, DFMO at the dose of 0.5 gm/M^2/d for 6 mo did not result in any cytological improvement and did not affect any of the secondary biomarkers tested, such as proliferating cell nuclear antigen expression (an index of cell proliferation), mammographic breast density, or serum insulin-like growth factor (IGF)-I and IGF-I/IGFBP-3 ratio. As recognized by the authors, however, these negative results may be explained by an insufficient dose of DFMO that, although able to modestly reduce average total urine polyamines, did not affect the spermidine/spermine ratio. The mean plasma level of DFMO was, indeed, found to be well below the level associated with decreased spermidine/spermine ratio and suppression of proliferation. Therefore, the merit of targeting the polyamine pathway in breast cancer chemoprevention remains to be tested using a more effective regimen.

3. Breast Cancer Cell Proliferation

Considerable evidence from our and other laboratories has shown that polyamines are critically involved in breast cancer cell proliferation *(21)*. In hormone-responsive tumors, ODC activity is stimulated by estrogen leading to an increased cellular polyamine content *(22)*. Administration of DFMO has been shown to consistently inhibit estradiol-stimulated ODC activity and estrogen-stimulated growth in numerous experimental systems *(23–25)*. It is important to emphasize, however, that polyamines are selectively involved in hormone action because other estrogen-regulated events (assessed concomitantly with tumor growth) have not been found to be affected by DFMO administration *(25)*. Polyamines are likely to influence hormone action in breast cancer cells at multiple steps. They include the association kinetics of the estrogen receptor with specific DNA sequences *(26)*, the synthesis of estradiol-regulated, cell-cycle specific genes *(27)* and the synthesis or action of hormonally regulated growth

factors *(24,28)*. Because ODC activity and cellular polyamine levels are increased in breast cancer compared with normal breast tissue *(10,11)*, we hypothesized that constitutive activation of the polyamine pathway could provide breast cancer cells with a growth advantage, possibly bypassing the need for estrogen stimulation and contributing to the acquisition of hormone independence. To directly test the influence of increased polyamine biosynthetic activity on phenotypical features of breast cancer cells, we induced ODC overexpression in the hormone-responsive, MCF-7 human breast cancer cell line. Although ODC-overexpressing clones exhibited a statistically significant increase in basal growth and a decreased sensitivity to the proliferative effects of estradiol (by approx 35%), these phenotypical changes were relatively modest and of dubious biological significance *(29)*. Analysis of cellular polyamine pools showed that ODC overexpression led to a marked and selective accumulation of putrescine, whereas levels of spermidine and spermine were not affected *(29)*. This profile is quite different from that found in human breast cancer specimens, in which all three polyamines are increased *(10,11)*.

In the attempt to increase the cellular levels of the more distal polyamines, we have transfected MCF-7 cells with an AdoMetDC-complimentary DNA *(30,31)*. AdoMetDC-overexpressing MCF-7 cells did not manifest any significant change in basal growth or in hormone responsiveness *(30,31)*. Their polyamine profiles, however, were also quite different from that of naturally occurring tumors and consisted of a selective increase in spermine, whereas putrescine and spermidine levels were both suppressed as a result of the compensatory decrease in ODC *(30,31)*.

In the aggregate, these results emphasize the difficulty in investigating the role of activation of polyamine biosynthesis in breast cancer biology by inducing overexpression of individual polyamine biosynthetic enzymes. As a consequence of the multiple compensatory events taking place when either ODC or AdoMetDC activity are artificially enhanced, the resulting polyamine profiles are quite different from those of actual human breast cancer specimens in which, evidently, these homeostatic mechanisms are lost, thus allowing accumulation of all three polyamines.

Despite abundant data in the literature supporting the antitumor action of DFMO in numerous experimental breast cancer systems, evidence indicating clinical efficacy in women with metastatic breast cancer is lacking. We participated in a phase I/II tolerability, pharmacokinetic, and efficacy study of high-dose DFMO (4800 mg po three times per day for 14 d, followed by a 2-wk drug holiday on a 28-d cycle) in 21 pretreated women with metastatic breast cancer *(32)*. This regimen was well tolerated without any clinically significant toxicity. DFMO treatment caused a modest-to-moderate reduction in urinary polyamine levels (only significant for putrescine) that persisted during the 2-wk drug holiday period. Although no patient achieved an objective remission, one woman with extensive, progressive liver and bone metastasis obtained disease stabilization in excess of 18 mo. Clearly, antipolyamine therapy in breast cancer needs to be tested in a group of patients with a more favorable disease using a more effective regimen in inducing polyamine suppression.

A drawback of DFMO treatment is its inability to effectively suppress the more distal polyamines, spermidine and particularly spermine, primarily because of the compensatory

increase in AdoMetDC activity. Therefore, inhibition of AdoMetDC activity has been proposed as an additional form of antipolyamine therapy. Among various AdoMetDC inhibitors, SAM486A (previously designated CGP48664) has been shown by us *(33)* and other investigators *(34)* to have antiproliferative activity in numerous experimental systems, including breast cancer. Under in vitro conditions, SAM486A administration induces a consistent suppression of cellular spermine associated with an increase in putrescine as a result of the compensatory increase in ODC activity *(33–35)*. In most cell lines, SAM486A has also been shown to lower spermidine *(33,34)*, although we have recently observed an increase in MDA-MB-435 breast cancer cells *(35)*. Under in vitro conditions, we have recently shown that combination treatment of MDA-MB-435 cells with DFMO and SAM486A exerted a greater suppression of cellular polyamine levels and a superior antiproliferative effect than the individual treatments *(35)*. SAM486A is being tested in phase I and II clinical trials *(36–38)*. It has been found to have acceptable toxicity and also promising clinical activity in patients with poor prognosis non-Hodgkin's lymphoma *(38)*. Despite its clinical use, however, virtually no information is available on changes in tumor polyamine levels in vivo induced by this compound. In two sequential tumor specimens obtained from a single patient before and after treatment, Siu et al. *(39)* reported a 70% reduction in spermine associated with a 16-fold increase in putrescine and a 2.63-fold increase in spermidine (changes similar to those found by us in MDA-MB-435 cells *[35]*).

To address the effect of SAM486A on breast cancer growth and polyamine levels in vivo, we performed a dose response study (0, 1, 5, 10 mg/kg ip daily) in nude mice orthotopically implanted with MDA-MB-435 breast cancer xenografts *(35)*. Unexpectedly, we found that the lowest dose, 1 mg/kg, was the most effective in suppressing tumor growth (Fig. 1A) and the only one able to reduce spermine (Fig. 1B). With increasing doses of the compound, we observed a progressive increase in spermidine and spermine, along with the expected major increase in putrescine resulting from the compensatory increase in ODC activity (Fig. 1B). We postulate that at least one of the mechanisms responsible for this observation may be the increased cellular accumulation of the precursor putrescine, which may override the block in AdoMetDC activity and allow the formation of the more distal polyamines. Whatever the mechanism, these results point to the importance of carefully evaluating the effects of SAM486A on cellular polyamine levels to identify the most effective dose. As probably is the case in many forms of biological therapy (as opposed to chemotherapy), the maximum tolerated dose is not necessarily the most effective.

4. Polyamines and Breast Cancer Progression and Metastasis

The natural history of human breast cancer is characterized by an inevitable transition from a relatively indolent hormone-dependent phenotype to a more aggressive typically hormone-independent status that is ultimately responsible for the patient's demise. Evidence indicates that activation of the polyamine pathway may be one of the contributing factors to breast cancer progression. Using an experimental system of mammary tumor progression, we found that an increase in cellular ODC activity was associated with transition to hormone independence and to a less differentiated and more metastatic phenotype *(40)*. With respect to human breast cancer, increased ODC

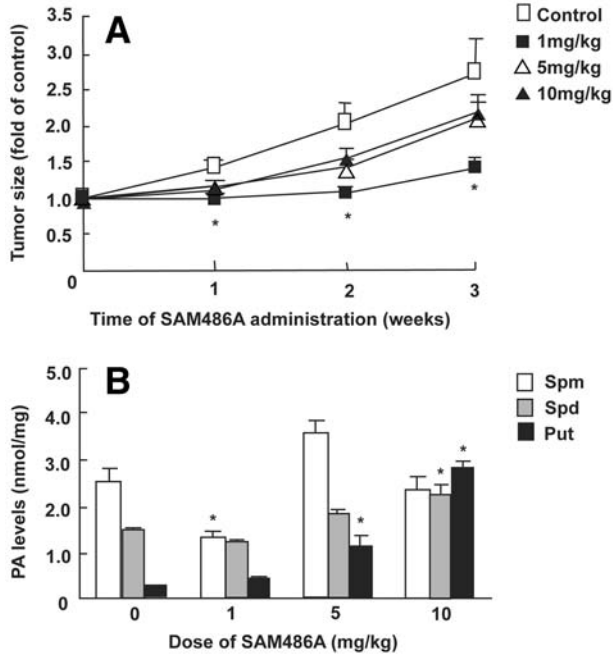

Fig. 1. Effect of SAM486A on tumor growth **(A)** and polyamine (PA) levels **(B)** in nude mice. A total of 5×10^6 cells suspended in Hank's balanced salt solution were implanted orthotopically into two axillary mammary fat pads per mouse of 4-wk-old female athymic nude mice. Two weeks later, when tumor areas averaged 80 mm^2, mice were randomly assigned to receive increasing doses of SAM486A (0, 1 mg/kg, 5 mg/kg, or 10 mg/kg) daily, by ip injection. Tumor growth was monitored once per week by measurement of tumor area (length × width) and expressed as fold increase over baseline **(A)**. After 3 wk of SAM486A administration, the mice were sacrificed, the tumors were removed and PA levels in the tumors were measured **(B)**. Statistical analysis: **(A)** $*p < 0.01$ vs control (analysis of variance followed by Dunnett's multiple comparison test). **(B)** $*p < 0.01$ vs control. (Reproduced with permission from ref. *35*.)

activity has been shown to be associated with adverse prognostic features such as high cellularity, low histological differentiation, high nuclear aplasia, peritumoral lymphatic and blood vessel invasion, high S-phase fraction, and cathepsin-D expression *(11,41)*.

In a pilot study involving a cohort of 50 primary human breast cancers, we have observed that ODC activity was a strong negative, independent prognostic factor of overall survival *(42)*. Figure 2 illustrates the effect of a 10-fold difference in ODC activity on overall survival after adjustment for other prognostic factors. This observation has been subsequently confirmed in a larger cohort of patients *(11)*. Because patients' survival is strictly related to the development of metastasis, this finding strongly suggests that increased ODC activity and hence, activation of polyamine biosynthesis, is involved in the metastatic process.

To address the involvement of polyamines in breast cancer invasiveness and metastasis, we turned to the hormone-independent MDA-MB-435 and MDA-MB-231

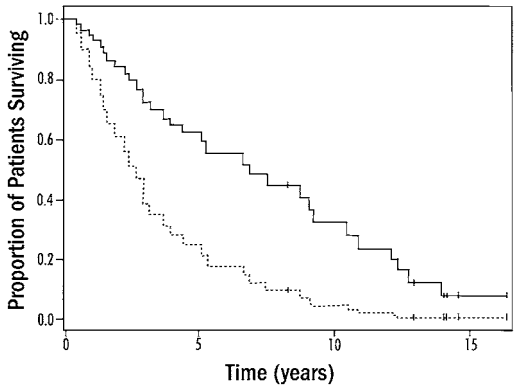

Fig. 2. Influence of a 10-fold difference in ODC (____, ODC = 10 pmol/mg; - - - - ODC = 100 pmol/mg) on breast cancer survival after adjustments were introduced for estrogen receptor and lymph nodal status. (Reproduced with permission from ref. *42*.)

human breast cancer cell lines which manifest an aggressive behavior both in vitro and in vivo. We found that DFMO administration, in addition to inhibiting cell proliferation, reduced invasiveness in matrigel of both cell lines by 70% *(43)*. Furthermore, DFMO treatment inhibited the ability of MDA-MB-435 cells to grow in a "branching" pattern in the matrigel outgrowth assay, which is a feature of aggressive tumors. More importantly, DFMO treatment significantly inhibited the development of pulmonary metastasis from MDA-MB-435 human breast cancer cells orthotopically implanted into the mammary fat pad of athymic nude mice *(43)*.

Using the same orthotopic model system, we next tested whether DFMO inhibits lung colonization by tumor cells or expansion of single-cell deposits into metastasis *(44)*. To address this issue, we took advantage of the availability of green fluorescent protein-tagged MDA-MB-435 cells, which can be tracked at the single-cell level throughout the entire lung. As shown in Table 1, DFMO treatment was again found to be effective in suppressing pulmonary metastasis. DFMO reduced the development of both multicellular and single-cell metastatic deposits, thus suggesting that its effect was primarily preventing lung colonization, rather than halting progression of single-cell deposits to overt metastasis. If the latter were true, one would have anticipated a higher single cell to multicellular metastasis ratio in the DFMO-treated mice. The findings reported in Table 1 also indicate that a more prolonged duration of treatment did not result in a superior antimetastatic effect, whereas discontinuation of DFMO was not associated with an increase in pulmonary metastasis, at least during the 4-wk period of observation. It is worth emphasizing that in this experimental system, the antimetastatic effect of DFMO was totally independent of any suppressive effect on the growth of the primary tumor. As a matter of fact, as shown in Fig. 3, the growth of the primary tumor was not affected at all by DFMO treatment, despite a complete suppression of putrescine and a 70% decrease in cellular levels of spermidine induced by DFMO.

To test the general applicability of these observations, in collaboration with Dr. Dan Welch at the University of Alabama Cancer Center, we are testing the effect of DFMO in

Table 1
Effects of the Different DFMO Treatment Regimens on Pulmonary, Lymph Nodal Metastasis, and Local Recurrence from GFP-Tagged MDA-MB-435 Breast Cancer Xenografts in Nude Mice

	Pulmonary metastasis			
		No. of metastasis/mouse[a]		
Experimental Groups	No. of mice with metastasis	Total	Multicellular deposits	Single-cell deposits
Control (n = 15)	14 (93%)[b]	7.3 ± 4.9[c]	4.3 ± 3.7[c]	3.0 ± 3.0[b]
DFMO (10 wk) (n = 11)	7 (64%)	3.5 ± 6.3	2.5 ± 4.7	1.0 ± 1.9
DFMO (10 wk) DFMO (4 wk) (n = 10)	6 (60%)	2.9 ± 3.7	1.8 ± 2.8	1.1 ± 1.7
DFMO (10 wk) Off (4 wk) (n = 12)	7 (58%)	1.9 ± 2.3	1.3 ± 2.0	0.6 ± 1.2

[a]Data represent means ± SD.
[b]p = 0.05 vs the combined DFMO treatment groups (10 and 14 wk).
[c]p < 0.05 vs each of the remaining groups.
(Reproduced with permission from ref. *44*.)

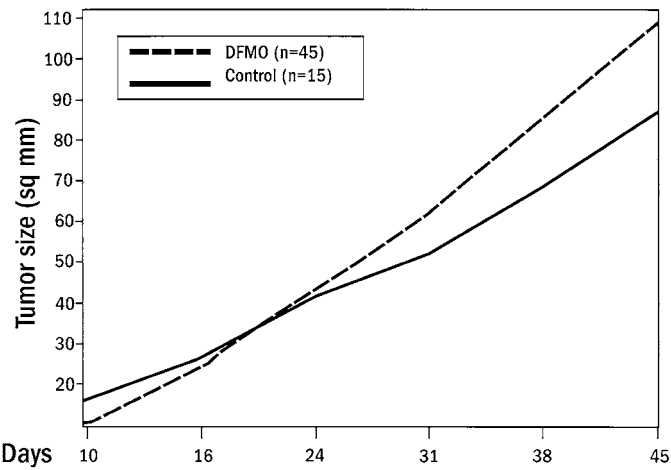

Fig. 3. Growth in nude mice of orthotopically implanted green fluorescent protein-tagged MDA-MB-435 breast cancer cells. A total of 1×10^6 cells in 0.1 mL Hank's balanced salt solution were injected in athymic mice in the absence and in the presence of DFMO treatment (2% in drinking H_2O). Growth curve analysis using a linear model revealed no statistically significant difference between control and DFMO treatment. (Reproduced from ref. *44* with permission of Springer Science and Business Media.)

different experimental systems of breast cancer metastasis. Preliminary unpublished data indicate that DFMO treatment significantly reduces skeletal metastasis from MDA-MB-435 cells injected in the left ventricle of the heart of athymic mice. In addition, DFMO administration also reduces the incidence of pulmonary metastasis in mice injected iv with MDA-MB-231 human breast cancer cells (unpublished observations).

As discussed previously, AdoMetDC may be an additional important target in the development of optimal antipolyamine therapy for prevention of metastasis. SAM486A has already been shown to have antimetastatic activity against melanoma in nude mice *(45)*. We are particularly interested in the combined inhibition of ODC and AdoMetDC to optimize polyamine depletion and antitumor action. We have recently observed that combined treatment with DFMO and SAM486A not only had a synergistic inhibitory effect on tumor cell proliferation, but also on invasiveness in matrigel *(35)*. We are testing whether the combined administration of these two compounds has an antimetastatic effect superior to the individual treatments.

5. Mechanism of Action of Antipolyamine Therapy

Figure 4 illustrates our ongoing research on the mechanisms of antitumor action of DFMO in breast cancer cells. We have shown that, in MDA-MB-435 cells, DFMO activates the MAPK pathway, as evidenced by increased phosphorylation of extracellular regulated kinase (ERK)-1 and ERK-2 *(46)*. A similar effect of DFMO has been reported by us in MCF-10A human breast epithelial cells *(47)* and by other investigators in intestinal epithelial and melanoma cells where this effect has been connected to induction of cell-cycle arrest *(48,49)*. We found that in MDA-MB-435 cells, activation of MAPK is causally linked to the anti-invasive action of DFMO because inhibition of ERK phosphorylation with the MEK inhibitor PD98059 reversed the effect of DFMO (Fig. 5) *(46)*. Our results, shown in Fig. 5, also indicate that the role of the MAPK pathway in invasiveness in our experimental system depends on the specific cellular context. In the absence of concomitant DFMO administration, PD98059 treatment either had no effect on invasiveness (Fig. 5A) or exerted an anti-invasive effect (Fig. 5B), depending on whether hepatocyte growth factor was present or absent as a chemoattractant.

The activation of the MAPK pathway by DFMO is difficult to reconcile with previous reports from our and other laboratories indicating that induction of ODC overexpression also leads to increased ERK-1 and -2 phosphorylation *(13,16,50)*. Clearly, the link between the polyamines and the MAPK pathway is complex and likely to vary, depending on the cell type and the specific stimulus to which the cells are exposed.

We have been interested in identifying mechanisms downstream of the MAPK pathway that may be responsible for the anti-invasive effect of DFMO in our system. We observed that activation of the MAPK pathway by DFMO induced a marked increase in thrombospondin-1 (TSP-1) production by MDA-MB-435 cells because the stimulation of TSP-1 by DFMO could be blocked by the MEK inhibitor PD98059 (Fig. 6) *(46)*. TSP-1 is an extracellular matrix glycoprotein that has been found to have predominantly an antimetastatic activity, including in MDA-MB-435 breast cancer cells *(51,52)*. It is thought that the tumor-suppressive function and antimetastatic action of TSP-1 is largely from its potent antiangiogenic effect *(53)*. Therefore, it is conceivable

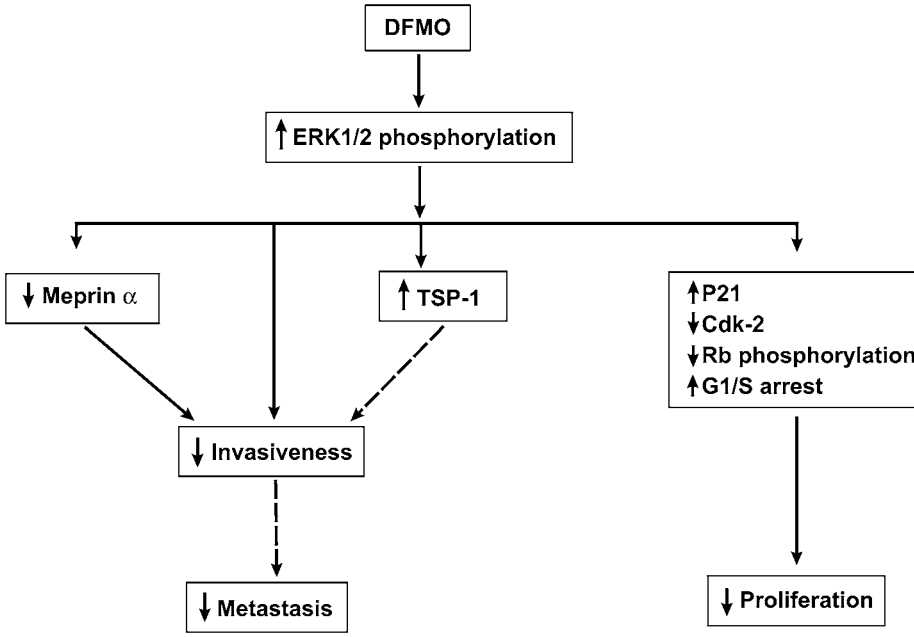

Fig. 4. Diagram summarizing the mechanism of antitumor action of DFMO in MDA-MB-435 human breast cancer cells. Activation of the mitogen-activated protein kinase pathway appears to be a central event mediating the effects of DFMO on meprin α, thrombospondin-1 (both likely to be involved in invasiveness and metastasis), and cell-cycle progression. Dotted lines indicate hypothetical connections, not yet proven experimentally.

that at least one of the mechanisms of antimetastatic action of DFMO in our system is through suppression of angiogenesis caused by upregulation of TSP-1 through the MAPK pathway. Obviously, it will be necessary to determine whether the effect of DFMO is blocked when TSP-1 production or action is inhibited before the increase in TSP-1 can be causally linked to the anti-invasive effect of DFMO.

Recent experiments conducted in collaboration with the laboratory of Dr. Judith Bond in the Department of Biochemistry and Molecular Biology at our institution have shown that expression of the metalloprotease, meprin α, is suppressed by DFMO through activation of the MAPK pathway because meprin α expression is restored in DFMO-treated MDA-MB-435 cells with administration of the MEK inhibitor, PD98059 *(54)*. Regulation of protease expression and activity can certainly be an important mechanism by which DFMO affects the invasive and metastatic properties of breast cancer cells. In other systems, protease expression has been shown to be elevated after induction of ODC overexpression *(55,56)* and inhibited in response to DFMO administration *(56)*.

Finally, in a recent series of yet-unpublished experiments conducted in MDA-MB-435 cells, we have shown that DFMO-induced ERK phosphorylation is causally linked to

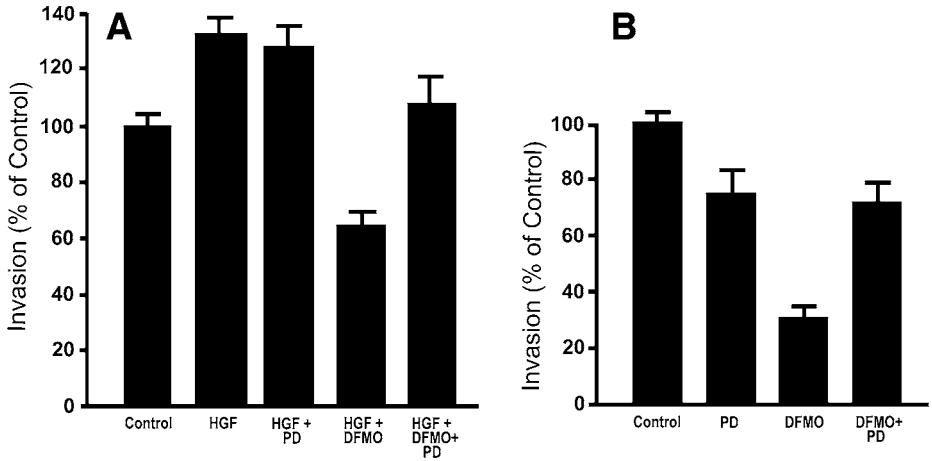

Fig. 5. Effect of PD98059 Parke-Davis (PD) on DFMO-induced suppression of invasiveness in the presence **(A)** or in the absence **(B)** of hepatocyte growth factor (HGF) (40 ng/mL) as a chemoattractant. MDA-MB-435 breast cancer cells were cultured in regular medium in the absence and in the presence of DFMO (1 m*M*) for 48 h before plating in the matrigel assay. PD treatment (50 μ*M*) was added 1 h before plating in the matrigel assay (time found to be sufficient to block extracellular regulated kinase phosphorylation) and continued for the duration of the experiment. Data are expressed as percent of control. Bars represent means ± standard error of the mean. The number of replicate experiments is five in **(A)** and four in **(B)**. Both in the presence **(A)** and in the absence of HGF **(B)**, treatment with PD significantly reversed the anti-invasive effect of DFMO ($p < 0.05$). (Reproduced from ref. *46* with permission of Springer Science and Business Media.)

Fig. 6. Effect of PD98059 Parke-Davis (PD) on DFMO-induced stimulation of thrombospondin-1 (TSP-1) production. MDA-MB-435 breast cancer cells were cultured for 48 h in regular medium in the absence and in the presence of DFMO. The cells were then stepped down to serum-free OPTI-MEM containing the same treatments for 48 h. PD98059 was added 1 h before transition to serum-free media conditions and continued until the end of the experiment. The conditioned media were collected, processed, and subjected to Western analysis using an antibody against TSP-1 as described (*46*). (Reproduced from ref. *46* with permission of Springer Science and Business Media.)

increased p21 expression, reduced cdk-2 protein and activity, reduction in the phosphorylation of the retinoblastoma gene, and G1/S arrest. These results are in general agreement with similar observations reported in melanoma and intestinal epithelial cells *(48,49)*.

6. Conclusions

There is considerable evidence supporting a critical role for polyamines in multiple aspects of human breast cancer biology. In the author's opinion, the most promising role for antipolyamine therapy is in the adjuvant setting when the tumor burden is low. An attractive feature of this treatment is its potential effectiveness in hormone-independent tumors that are more aggressive and have fewer treatment options than the hormone-dependent ones. Our preliminary data, showing a strong antimetastatic effect of DFMO in multiple experimental breast cancer systems, are supportive of the possible clinical application of antipolyamine therapy. Based on preclinical evidence, it is also plausible that antipolyamine therapy may play a role in breast cancer prevention. Obviously, a major challenge remains to devise an effective and safe antipolyamine regimen that can be used in humans. It is clear that polyamines interact with multiple other signaling pathways to influence the breast cancer phenotype. In addition to the MAPK pathway, polyamine depletion has also been shown to activate p38, Jun-N-terminal kinase, and signal transducers and activators of transcription signaling *(35)*. However, the role of these effects in mediating the biological actions of polyamines is unknown. It will be critical to identify which signaling molecules cooperate with the polyamine pathway to rationally devise biological treatments that can be used in concert with antipolyamine therapy.

Acknowledgment

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References

1. Gradishar, W. J. and Jordan, V. C. (1999) Pharmacology and use of antiestrogens in treatment and chemoprevention of breast cancer. In: *Endocrinology of Breast Cancer* (Manni, A., ed.), Humana Press, Inc., Totowa, NJ, pp. 283–311.
2. Harvey, H. (1999) Aromatase inhibitors in breast carcinoma. In: *Endocrinology of Breast Cancer* (Manni, A., ed.), Humana Press, Inc., Totowa, NJ, pp. 233–243.
3. Pegg, A. E. (1988) Polyamine metabolism and its importance in neoplastic growth and a target for chemotherapy. *Cancer Res.* **48,** 759–774.
4. Manni, A. (2002) Polyamine involvement in breast cancer phenotype. *In Vivo* **16,** 493–500.
5. Scalabrino, G. and Ferioli, M. E.(1982) Polyamines in mammalian tumors. Part II. *Adv. Cancer Res.* **36,** 1–102.
6. Scalabrino, G. and Ferioli, M. E. (1981) Polyamines in mammalian tumors. Part I. *Adv. Cancer Res.* **35,** 151–268.
7. Luk, G. D. and Baylin, S. B. (1984) Ornithine decarboxylase as a biologic marker in familial colonic polyposis. *N. Engl. J. Med.* **311,** 80–83.
8. O'Brien, T. G. (1976) The induction of ornithine decarboxylase as an early, possibly obligatory, event in mouse skin carcinogenesis. *Cancer Res.* **36,** 2644–2653.
9. Kingsnorth, A. N., King, W. W., Diekema, K. A., McCann, P. P., Ross, J. S., and Malt, R. A. (1983) Inhibition of ornithine decarboxylase with 2-difluoromethylornithine: reduced incidence of dimethylhydrazine-induced colon tumors in mice. *Cancer Res.* **43,** 2545–2549.

10. Persson, L. and Rosengren, E. (1989) Increased formation of N1-acetylspermidine in human breast cancer. *Cancer Lett.* **45,** 83–86.
11. Canizares, F., Salinas, J., de las Heras, M., et al. (1999) Prognostic value of ornithine decarboxylase and polyamines in human breast cancer: correlation with clinicopathologic parameters. *Clin. Cancer Res.* **5,** 2035–2041.
12. Manni, A., Astrow, S. H., Gammon, S., Thompson, J., Mauger, D., and Washington, S. (2001) Immunohistochemical detection of ornithine-decarboxylase in primary and metastatic human breast cancer specimens. *Breast Cancer Res. Treat.* **67,** 147–156.
13. Manni, A., Wechter, R., Verderame, M. F., and Mauger, D. (1998) Cooperativity between the polyamine pathway and HER-2neu in transformation of human mammary epithelial cells in culture: role of the MAPK pathway. *Int. J. Cancer* **76,** 563–570.
14. Smith, M. K., Trempus, C. S., and Gilmour, S. K. (1998) Co-operation between follicular ornithine decarboxylase and v-Ha-ras induces spontaneous papillomas and malignant conversion in transgenic skin. *Carcinogenesis* **19,** 1409–1415.
15. Sivaraman, V. S., Wang, H., Nuovo, G. J., and Malbon, C. C. (1997) Hyperexpression of mitogen-activated protein kinase in human breast cancer. *J. Clin. Invest.* **99,** 1478–1483.
16. Kubota, S., Kiyosawa, H., Nomura, Y., Yamada, T., and Seyama, Y. (1997) Ornithine decarboxylase overexpression in mouse 10T1/2 fibroblasts: cellular transformation and invasion. *J. Natl. Cancer Inst.* **89,** 567–571.
17. Thompson, H. J., Meeker, L. D., Herbst, E. J., Ronan, A. M., and Minocha, R. (1985) Effect of concentration of D,L-2-difluoromethylornithine on murine mammary carcinogenesis. *Cancer Res.* **45,** 1170–1173.
18. Green, J. E., Shibata, M. A., Shibata, E., et al. (2001) 2-difluoromethylornithine and dehydroepiandrosterone inhibit mammary tumor progression but not mammary or prostate tumor initiation in C3(1)/SV40 T/t-antigen transgenic mice. *Cancer Res.* **61,** 7449–7455.
19. Meyskens, F. L., Jr. and Gerner, E. W. (1999) Development of difluoromethylornithine (DFMO) as a chemoprevention agent. *Clin. Cancer Res.* **5,** 945–951.
20. Fabian, C. J., Kimler, B. F., Brady, D. A., et al. (2002) A phase II breast cancer chemoprevention trial of oral alpha-difluoromethylornithine: breast tissue, imaging, and serum and urine biomarkers. *Clin. Cancer Res.* **8,** 3105–3117.
21. Manni, A. (1999) Role of the polyamine pathway in the natural history of breast cancer. In: *Contemporary Endocrinology: Endocrinology of Breast Cancer* (Manni, A., ed.), Humana Press, Inc., Totowa, NJ, p. 221–230.
22. Thomas, T. and Thomas, T. J. (1994) Estradiol control of ornithine decarboxylase mRNA, enzyme activity, and polyamine levels in MCF-7 breast cancer cells: therapeutic implications. *Breast Cancer Res. Treat.* **29,** 189–201.
23. Manni, A. and Wright, C. (1984) Polyamines as mediators of estrogen action on the growth of experimental breast cancer in rats. *J. Natl. Cancer Inst.* **73,** 511–514.
24. Glikman, P. L., Manni, A., Bartholomew, M., and Demers, L. (1990) Polyamine involvement in basal and estradiol-stimulated insulin-like growth factor I secretion and action in breast cancer cells in culture. *J. Steroid Biochem. Mol. Biol.* **37,** 1–10.
25. Manni, A., Badger, B., Lynch, J., and Demers, L. (1991) Selectivity of polyamine involvement in hormone action on normal and neoplastic target tissues of the rat. *Breast Cancer Res. Treat.* **17,** 187–196.
26. Thomas, T. (1989) Structural specificity of polyamines in facilitating the high affinity binding of estrogen receptor to specific DNA sequences. *FASEB J.* **3,** A1192.
27. Thomas, T. and Thomas, T. J. (1994) Regulation of cyclin B1 by estradiol and polyamines in MCF-7 breast cancer cells. *Cancer Res.* **54,** 1077–1084.

28. Manni, A., Wright, C., Feil, P., et al. (1986) Autocrine stimulation by estradiol-regulated growth factors of rat hormone-responsive mammary cancer: interaction with the polyamine pathway. *Cancer Res.* **46,** 1594–1598.
29. Manni, A., Wechter, R., Wei, L., Heitjan, D., and Demers, L. (1995) Phenotypic features of breast cancer cells overexpressing ornithine-decarboxylase. *J. Cell. Physiol.* **163,** 129–136.
30. Manni, A., Badger, B., Grove, R., Kunselman, S., and Demers, L. (1995) Isolation and characterization of human breast cancer cells overexpressing S-adenosylmethionine decarboxylase. *Cancer Lett.* **95,** 23–28.
31. Manni, A., Fischer, S., Franks, M., et al. (2001) S-adenosylmethionine decarboxylase overexpression reduces invasiveness and tumorigenicity in nude mice of MCF-7 breast cancer cells. *Int. J. Oncol.* **19,** 317–323.
32. O'Shaughnessy, J. A., Demers, L. M., Jones, S. E, et al. (1999) Alpha-difluoromethylornithine as treatment for metastatic breast cancer patients. *Clin. Cancer Res.* **5,** 3438–3444.
33. Manni, A., Badger, B., Wechter, R., Kunselman, S., Rossini, A., and Demers, L. (1995) Biochemical and growth-modulatory effects of the new S-adenosylmethionine decarboxylase inhibitor CGP 48664 in malignant and immortalized normal human breast epithelial cells in culture. *Int. J. Cancer* **62,** 485–491.
34. Regenass, U., Mett, H., Stanek, J., Mueller, M., Kramer, D., and Porter, C. W. (1994) CGP 48664, a new S-adenosylmethionine decarboxylase inhibitor with broad spectrum antiproliferative and antitumor activity. *Cancer Res.* **54,** 3210–3217.
35. Hu, X., Washington, S., Verderame, M. F., Demers, L. M., Mauger, D., and Manni, A. (2004) Biological activity of the S-adenosylmethionine decarboxylase inhibitor SAM486A in human breast cancer cells in vitro and in vivo. *Int. J. Oncol.* **25,** 1831–1838.
36. Paridaens, R., Uges, D. R., Barbet, N., et al. (2000) A phase I study of a new polyamine biosynthesis inhibitor, SAM486A, in cancer patients with solid tumours. *Br. J. Cancer* **83,** 594–601.
37. van Zuylen, L., Bridgewater, J., Sparreboom, A., et al. (2004) Phase I and pharmacokinetic study of the polyamine synthesis inhibitor SAM486A in combination with 5-fluorouracil/leucovorin in metastatic colorectal cancer. *Clin. Cancer Res.* **10,** 1949–1955.
38. Pless, M., Belhadj, K., Menssen, H. D., et al. (2004) Clinical efficacy, tolerability, and safety of SAM486A, a novel polyamine biosynthesis inhibitor, in patients with relapsed or refractory non-Hodgkin's lymphoma: results from a phase II multicenter study. *Clin. Cancer Res.* **10,** 1299–1305.
39. Siu, L. L., Rowinsky, E. K., Hammond, L. A., et al. (2002) A phase I and pharmacokinetic study of SAM486A, a novel polyamine biosynthesis inhibitor, administered on a daily-times-five every-three-week schedule in patients with Advanced solid malignancies. *Clin. Cancer Res.* **8,** 2157–2166.
40. Manni, A., Grove, R., Kunselman, S., and Aldaz, M. (1995) Involvement of the polyamine pathway in breast cancer progression. *Cancer Lett.* **92,** 49–57.
41. Glikman, P., Vegh, I., Pollina, M. A., Mosto, A. H., and Levy, C. M. (1987) Ornithine decarboxylase activity, prolactin blood levels, and estradiol and progesterone receptors in human breast cancer. *Cancer* **60,** 2237–2243.
42. Manni, A., Mauger, D., Gimotty, P., and Badger, B. (1996) Prognostic influence on survival of increased ornithine decarboxylase activity in human breast cancer. *Clin. Cancer Res.* **2,** 1901–1906.
43. Manni, A., Washington, S., Griffith, J. W., et al. (2002) Influence of polyamines on in vitro and in vivo features of aggressive and metastatic behavior by human breast cancer cells. *Clin. Exp. Metastasis* **19,** 95–105.

44. Manni, A., Washington, S., Craig, L., et al. (2003) Effects of alpha-difluoromethylornithine on local recurrence and pulmonary metastasis from MDA-MB-435 breast cancer xenografts in nude mice. *Clin. Exp. Metastasis* **20,** 321–325.
45. Gutman, M., Beltran, P. J., Fan, D., et al. (1995) Treatment of nude mice with 4-amidinoindan-1-one2′-amidinohydrazone, a new S-adenosylmethionine decarboxylase inhibitor, delays growth and inhibits metastasis of human melanoma cells. *Melanoma Res.* **5,** 147–154.
46. Manni, A., Washington, S., Mauger, D., Hackett, D. A., and Verderame, M. F. (2004) Cellular mechanisms mediating the anti-invasive properties of the ornithine decarboxylase inhibitor alpha-difluoromethylornithine (DFMO) in human breast cancer cells. *Clin. Exp. Metastasis* **21,** 461–467.
47. Manni, A, Trout, D., Verderame, M. F., Washington, S., Mauger, D., and Demers, L. (2001) Effect of alpha-difluoromethyl-ornithine on the expression and function of the epidermal growth factor receptor in human breast epithelial cells in culture. *Breast Cancer Res. Treat.* **68,** 139–146.
48. Chen, Y., Alm, K., Vujcic, S., et al. (2003) The role of mitogen-activated protein kinase activation in determining cellular outcomes in polyamine analogue-treated human melanoma cells. *Cancer Res.* **63,** 3619–3625.
49. Ray, R. M., Zimmerman, B. J., McCormack, S. A., Patel, T. B., and Johnson, L. R. (1999) Polyamine depletion arrests cell cycle and induces inhibitors p21(Waf1/Cip1), p27(Kip1), and p53 in IEC-6 cells. *Am. J. Physiol.* **276,** C684–C691.
50. Manni, A., Wechter, R., Gilmour, S., Verderame, M. F., Mauger, D., and Demers, L. M. (1997) Ornithine decarboxylase over-expression stimulates mitogen-activated protein kinase and anchorage-independent growth of human breast epithelial cells. *Int. J. Cancer* **70,** 175–182.
51. Zabrenetzky, V., Harris, C. C., Steeg, P. S., and Roberts, D. D. (1994) Expression of the extracellular matrix molecule thrombospondin inversely correlates with malignant progression in melanoma, lung and breast carcinoma cell lines. *Int. J. Cancer* **59,** 191–195.
52. Weinstat-Saslow, D. L., Zabrenetzky, V. S., VanHoutte, K., Frazier, W. A., Roberts, D. D., and Steeg, P. S. (1994) Transfection of thrombospondin 1 complementary DNA into a human breast carcinoma cell line reduces primary tumor growth, metastatic potential, and angiogenesis. *Cancer Res.* **54,** 6504–6511.
53. Bagavandoss, P. and Wilks, J. W. (1990) Specific inhibition of endothelial cell proliferation by thrombospondin. *Biochem. Biophys. Res. Commun.* **170,** 867–872.
54. Matters, G. L., Manni, A., and Bond, J. S. (2004) Inhibition of polyamine synthesis by alpha-difluoromethylornithine (DFMO) reduces expression of the metalloprotease meprin alpha in human breast cancer cells. *Proc. 95th Ann. Meeting Am. Assoc. Cancer Res. Meeting* **45,** 426.
55. Smith, M. K., Goral, M. A., Wright, J. H., et al. (1997) Ornithine decarboxylase overexpression leads to increased epithelial tumor invasiveness. *Cancer Res.* **57,** 2104–2108.
56. Wallon, U. M., Shassetz, L. R., Cress, A. E., Bowden, G. T., and Gerner, E. W. (1994) Polyamine-dependent expression of the matrix metalloproteinase matrilysin in a human colon cancer-derived cell line. *Mol. Carcinog.* **11,** 138–144.

9

Polyamines in Regulation of Prostatic Cell Growth

Raymond G. Schipper, Vincent Cuijpers, Johannes C. Romijn, and Albert A. J. Verhofstad

1. Introduction

The prostate and prostate secretions have played an important role in the initial identification of polyamines. One of the polyamines, spermine, was reported in human semen as early as the 17th century by Antoni Van Leeuwenhoek. After the elucidation of the chemical structure of polyamines, about 200 yr later, the biochemical mechanism of action of polyamines in the prostate gland and semen has been studied extensively. Apart from this historical perspective, the prostate has one of the highest polyamine concentrations of any tissue. In rats, the amount of polyamines, especially spermidine, is highest in the ventral prostate followed by the dorsal and lateral prostate, whereas the coagulating glands and seminal vesicles show low values *(1)*. The human prostate gland synthesizes exceptionally high levels of spermine on an average of 130 mg/100 g per day *(2)*.

Growth and function of the prostate gland is controlled by androgens that are responsible for the development of male characteristics, such as hair and beard growth. The most important androgen in men is testosterone. Testosterone, produced primarily in the testis, can diffuse freely into prostate cells, where it is rapidly and irreversibly converted to its metabolically more active form, dihydrotestosterone (DHT), through the activity of the 5-α reductase. Increased DHT levels are suspected to promote DNA synthesis and replication of prostate cells.

One of the effects of testosterone stimulation on prostatic cells is the production of polyamines *(3–5)*. The activities of the polyamine biosynthetic enzymes ornithine decarboxylase (ODC), *S*-adenosylmethionine decarboxylase (AdoMetDC), and spermidine synthase (SpdS) are induced by androgens in a coordinated way, and expression of these enzymes is primarily localized to the glandular epithelial cells of the prostate *(5–7)*.

Studies on the murine *ODC* gene revealed that the ODC promoter contains an androgen-responsive element-like sequence that can bind to the androgen receptor in vitro *(6)*. Differential display studies in LNCaP cells showed that androgen regulation

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of ODC is directly mediated through the androgen receptor with kinetics of induction similar to that for prostate-specific antigen (PSA) *(7)*. Inhibition of ODC activity with difluoromethylornithine reduces the size of prostatic acini in the developing prostate, retards testosterone-induced regrowth of the prostate in castrated rats, and inhibits insulin-like growth factor 1-induced growth of the prostate *(8)*.

It is apparent that functions of ODC and polyamines in the prostate are related to cellular proliferation and secretory activities. The exact role of polyamines in prostate biology is not yet known, however, and needs further investigation. It has been postulated that spermine is involved in the maintenance of the functional secretory state of prostatic epithelium (*9*, and references herein). Interestingly, the (rat) prostate contains an androgen-regulated protein that has high affinity toward spermine (i.e., spermine-binding protein) *(10)*. Spermine-binding protein is phosphorylated by cyclic-adenosine monophosphate-independent protein kinases *(11)*.

The functional significance of seminal polyamines is a matter of debate. Studies of human prostatic fluids indicate that spermine is linked to citrate, also present in remarkably high concentrations in the prostate *(12)*. Complex formation with the negatively charged acid citrate would enable polyamines to penetrate in spermatozoa. Spermine has been localized in the middle and top parts of the acrosome, and possibly modulates sperm fertilization competence and the acrosomal reaction *(13)*. However, spermine present in sperm cells may also originate from endogenous polyamine biosynthesis because ODC has been shown to be involved in spermatogenesis *(14)*. Seminal polyamines may have other functions in regulation of seminal clotting or prevention of bacterial growth in the urinary tract *(15)*.

One might expect that prostatic cells compared with other cell types exhibit different mechanisms for regulation of their polyamines because they produce high levels of polyamines. Indeed, polyamine uptake and accumulation is significantly higher in prostate cancer cells compared with nonprostate cancer cell lines *(16)* because of a unique insensitivity of polyamine transport to regulation by polyamines or polyamine analogs in prostatic (cancer) cells *(17)*. A possible intriguing therapeutic implication of these findings is that exposure to polyamine antimetabolites (e.g., polyamine analogs) would induce downregulation of drug uptake in normal tissues, whereas at the same time, drug uptake is ensured to growth inhibitory levels in the prostate tumor or tissue.

The importance of polyamines in prostatic growth and differentiation has prompted studies to evaluate the clinical relevance of the ODC/polyamine system in prostate cancer, as will be discussed later in this chapter.

2. Polyamines and Cell Kinetic Behavior (Apoptosis, Proliferation) of Prostatic Cells

Prostate growth depends on the balance between death (apoptosis) and proliferation of prostatic cells. To investigate the role of polyamine metabolism in prostatic growth, we have monitored the levels of polyamines, as well as the activities of the polyamine biosynthetic enzyme, ODC, and the polyamine-catabolizing enzyme, Spd/Spm acetyltransferase (SSAT), in castration-induced regression and testosterone-stimulated regrowth of the rat prostate.

Before castration, both proliferation rate and apoptotic rate in the glandular prostatic tissue were less than 2%. In the first hours after castration, the apoptotic rate increased, reaching a maximum level of 15 to 20% after 2 d (Fig. 1A). Concomitantly, ODC activity (Fig. 1B) and polyamine levels (Fig. 1C) dramatically decreased, whereas SSAT activity progressively increased in the prostatic tissue (Fig. 1B). Regeneration of the prostate after suppletion of androgens coincided with a marked increase in ODC activity and recovery of polyamine concentrations to normal limits (Fig. 1A,C, right side). The effect of androgen/suppletion on prostate cell kinetics and polyamine parameters was notably less dramatic in the dorsolateral lobe of the prostate compared with the ventral lobe (results not shown).

Much ambiguity exists on the role of ODC in apoptosis because ODC activity is reported to be increased, decreased, or unchanged depending on the apoptotic stimulus or cell system used (*see* refs. *18* and *19*, and references herein). Remarkably, in most of these studies, ODC increases early during apoptosis but eventually polyamine levels mostly decreased. Several studies imply, however, that the increase in ODC activity is merely a trophic response to stress or is even just an epiphenomenon (*see* ref. *18* and references herein). The inability of ODC to maintain elevated polyamine levels may be owing to a precocious degradation of ODC during the cell death process. Proteolytic degradation of ODC is regulated by a unique protein, the so-called antizyme (AZ), which binds to ODC and facilitates its breakdown by the cytosolic 26S proteosome. In our recent study on the localization of ODC and AZ, we found that treatment of prostatic cancer cells with 1 m*M* putrescine resulted in nuclear induction of AZ that colocalized with a fusion protein of ODC conjugated with green fluorescent protein *(*GFP) *(20)*. Consistent with this is that the proteolytic breakdown observed during apoptosis mostly affects proteins needed for cell cycle and survival. Because polyamines are critically involved in cell-cycle control, ODC may be one of the proteins that are degraded by apoptosis-related proteases. No evidence has yet been found for the proteolysis of ODC by caspases. However, a potential cleavage site (YVAD) for caspases is present in ODC in most animals. Interestingly, the amino acid sequence of the very stable ODC of *Trypanosoma brucei* contains HVAD, a site less vulnerable to caspase-mediated proteolysis. It is therefore tempting to speculate that ODC is a potential caspase target.

Concomitant to castration-induced apoptosis, SSAT activity progressively increased in the prostatic tissue (Fig. 1B). We observed a similar, relatively late induction of SSAT in calcium-ionophore-induced apoptosis of prostate cancer cells *(21)*. These results suggest that polyamine catabolism is associated with the later stages of apoptosis.

Increased expression of SSAT during apoptosis has also been reported in other studies (*see* ref. *19* and references herein) suggesting that stimulation of polyamine catabolism is a general cellular response in cell death. Apoptosis induced by polyamine analogs has been associated with highly increased SSAT levels (*see* Chapters 12 and 15).

The link between SSAT induction and apoptosis may actually be associated with the action of another polyamine catabolic enzyme, polyamine oxidase, which oxidizes acetylated polyamines generated by SSAT and releases toxic byproducts, such as hydrogen peroxide and aldehydes (*see* Chapter 12), which in turn, could trigger the apoptotic process.

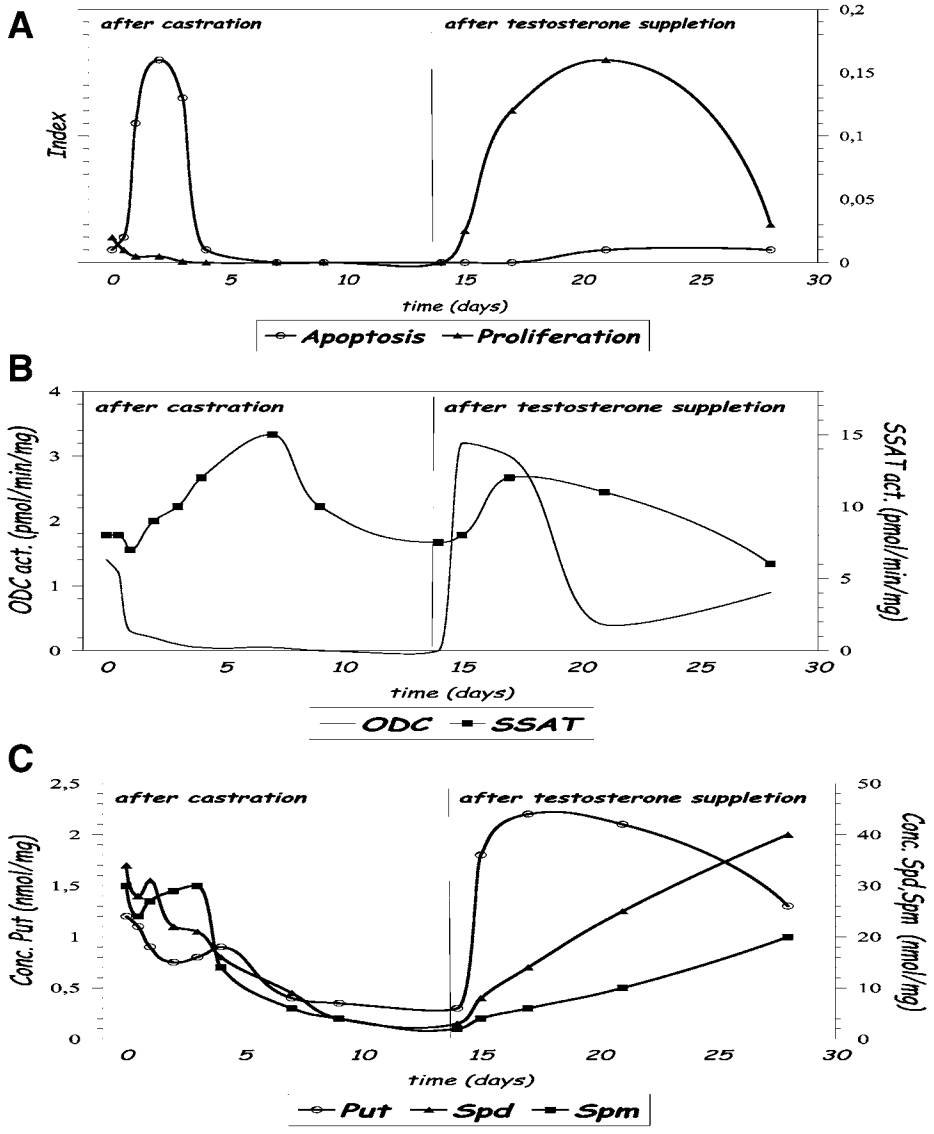


Fig. 1. Apoptosis and proliferation index, and parameters of polyamine homeostasis in castration-induced involution and testosterone-induced regrowth of the rat ventral prostate. **(A)** The apoptosis index was expressed as the fraction of *in situ* nick translation (ISNT)-positive glandular epithelial cells, as well as the proliferation index as the fraction of BrdU positive cells. **(B)** Activities of the polyamine biosynthetic enzyme ornithine decarboxylase (ODC) and the polyamine catabolic enzyme Spd/Spm N^1-acetyltransferases (SSAT). **(C)** Concentrations of putrescine (Put), spermidine (Spd), and spermine (Spm).

3. Polyamine Metabolism is Disturbed in Prostate Cancer

The recent development of the microarray technology has allowed analysis of expression levels of thousands of genes in one experiment. In a study to identify genes and pathways that are differentially expressed between androgen-dependent and androgen-independent cancer cells, Jenster et al. from the Department of Urology of the Erasmus MC in Rotterdam performed microarray experiments, using GeneChips that harbored approx 4000 known genes and 3000 expressed sequence tags. On each chip, two batches of RNA were compared by competitive hybridization with the glass-spotted complimentary DNAs. Comparing the expression of *ODC*, *AdoMetDC*, and *SSAT* genes in androgen-responsive LNCaP cells cultured in the absence or presence of 1 n*M* dihydrotestosterone (DHT) for 16 h, it was observed that androgen stimulated the expression of these enzymes twofold to fourfold (Jenster et al., unpublished).

In a recent study, Rhodes et al. *(22)* conducted a meta-analysis of four independent microarray datasets to identify consistently significant candidate genes for prostate cancer. This study revealed a consistent and significant deregulation of polyamine biosynthesis in prostate cancer. Specifically, they found that enzymes linked to polyamine synthesis (aspartate transaminase, aminoacylase, ODC, and spermidine synthase) were overexpressed in prostate cancer, whereas ornithine aminotransferase was underexpressed. The net effect is an increased biosynthesis of polyamines. Biologically this makes sense because polyamines have been implicated in cancer cell proliferation, protection from apoptosis, and DNA–protein binding.

To investigate if ODC is involved in the cell kinetic behavior of human prostatic cancer cells, we examined ODC in cultured cells or xenografts of human prostatic cell lines. ODC protein was determined by Western blotting according to Schipper et al. *(23)*. Activity of ODC was measured, as described earlier *(24)*, in 48-h cultures and in xenografts of the prostatic cancer cell lines.

Results, as summarized in Table 1, indicate that ODC protein, as well as ODC activity, correlate with the growth rate of prostatic cancer cells (i.e., the amount of ODC protein is highest in the relatively fast growing tumor lines PC-324, PC-374, and PC-346). This is in agreement with studies in rat prostate-derived Dunning tumors reporting a substantially higher ODC activity in the faster growing sublines *(25)*. Immunoblot analysis of human prostate cancer tissue specimens showed a significantly elevated ODC protein expression in the cancerous tissues as compared with the benign tissues *(26)*. Moreover, studies on the expression levels of the *ODC* gene in a series of 23 human prostate cancers dissected from radical prostatectomy specimens revealed significantly higher ODC mRNA levels in tumors compared with the benign tissue *(27)*. With a 5-yr follow-up study performed on the same cohort of patients, *ODC* gene profiling was proven to be an effective method for predicting the recurrence of prostate cancer, especially when combined with clinical–pathological parameters *(28)*.

4. Polyamines as Biomarkers for Prostate Cancer

The observation that not only a number of enzymatic steps in polyamine synthesis, but also enzymes directing substrates toward polyamines, are overexpressed in

Table 1
ODC/Polyamines in Different Phenotypes of Human Prostatic Cancer Cells

Cell line	Origin	Androgen dependent	AR pathway	Diff. grade	Td (d)	ODC protein	ODC activity (pmol/min/mg)	Spd (nm/mg)	Spm (nm/mg)	Spd/Spm ratio
PC-295	LN	Yes	Intact	++	18	+		0.64	1.41	0.32
PC-310	PC	Yes	Intact	++	13	+		0.32	1.45	0.22
PC-324	TUR	No	No AR	–	10	++		0.61	0.57	1.07
PC-339	TUR	No	No AR	–	20	+		0.81	0.49	1.65
PC-374	Skin	Sensitive	Intact	+	10	++		0.30	1.41	0.21
PC-346	TUR	Yes	Intact	++	7	+++		0.70	1.81	0.39
PC-3	Bone	No	No AR	–	12			4.8	4.1	1.17
PC3-C	PC-3	No	No AR		1.3		18.4	19.8	12.5	1.58
Du-145	Brain	No	No AR	±	15		2.8	2.1	1.6	1.31
DU-145-C	DU-145	No	No AR		2.5		7.7	5.2	4.3	1.20

Results are the mean of at least two different experiments. Variability between duplicate determinations was within 10% for the data shown here. PC, primary prostate tumor; LN, lymph node; TUR, transurethral resection; AR, androgen receptor; Td, tumor doubling time; -C, in vitro cell line; Diff., differentiation; Spd, spermidine; Spm, spermine.

prostatic cancer implies that polyamines may be suitable markers for prostate cancer. In the following sections, studies on the use of polyamines in body fluids and tissues as biomarkers for prostatic cancer are discussed.

4.1. Circulating Polyamines as Markers and Tracers for Prostatic Malignancies

Polyamines or their acetylated forms can also be secreted by cells and released into the circulation. Subsequently, circulating polyamines and their acetylated products can be taken up and reused by polyamine-demanding cells. Transport of polyamines from one cell to another via body fluids is believed to be carrier-mediated by either proteins (e.g., anti-polyamine antibodies, lipoproteins) or by cells (peripheral blood cells). Moulinoux et al. showed that more than 95% of circulating spermidine and spermine are transported by red blood cells (RBCs) and that RBC polyamine levels correlate with tumor progression in tumor-grafted animals. Results from these animal studies and investigations in patients suggest that RBC polyamine levels reflect the proliferation status of normal tissues and tumors including prostate carcinomas *(29,30)*.

As demonstrated by these studies, interorgan polyamine transport is realized by means of circulating polyamines, which participate in supporting normal and malignant cell proliferation. Because neoplastic growth requires high concentrations of polyamines, radioactively labeled polyamines have been used as tracers for imaging and characterization of cancers. Clark and Fair *(31)* reported that ^{14}C-putrescine, when injected in rats, preferably accumulated in the prostate. In vitro and in vivo uptake of radiolabeled ^{14}C-polyamines by rat prostate-derived Dunning tumor lines was investigated by Heston et al. *(32,33)*. These studies indicated that both labeled putrescine and spermidine are imported in cultured cells whereas, in tumor-bearing animals, labeled putrescine and cadaverine, but not spermidine and spermine were taken up by the tumor. Uptake of ^{14}C-putrescine by the rat prostatic tumor was higher than in the dorsolateral prostate. In contrast, in the ventral prostate uptake equals the amount taken up by the tumor. Pretreatment with difluoromethylornithine and androgen stimulation enhanced the uptake of ^{14}C-putrescine in the tumor but not in other tissues.

More recently, the potential of labeled putrescine as tracer for cancers has been studied using positron emission tomography (PET). PET-scan imaging of the Dunning R3327H prostatic carcinoma demonstrated a high tumor uptake of the labeled polyamine *N*-(3-[18F]fluoro-propyl)putrescine *(34,35)*. Uptake of ^{11}C-putrescine using PET-scan imaging has also been examined in brain tumors showing similar results *(36)*. However, PET studies in humans showed that the uptake of ^{11}C-putrescine in the prostates of normal controls exceeds uptake in prostates of patients with localized prostatic cancer *(37)*. These results suggest that ^{11}C-putrescine is not useful to trace human prostate adenocarcinoma.

4.2. Spermine as a Biomarker for Malignant Behavior of Prostatic Cancer

Spermine is present in the prostate in high concentrations (10–20 m*M*). Several studies suggest that a reduction of spermine levels may result from shifts in cellular behavior of prostatic (cancer) cells *(38–40)*.

Polyamine measurements in our panel of cultured cells or xenografts of human prostatic cell lines showed that the less differentiated cell lines (e.g., PC-324, PC-339) contained clearly less spermine than the tumor cell lines with differentiation characteristics resembling (androgen dependence, androgen receptor content, specific marker expression) more normal prostate epithelium (e.g., PC-295, PC-310, PC-346, and PC-374) (*see* Table 1).

These observations prompted us to search for a nondestructive tool to evaluate polyamine levels in biopsies and in vivo in the prostate *(38)*. Such a tool would be very useful to determine the predictive value of the polyamine content in prostate cancer as a marker for its biological behavior. Proton magnetic resonance spectroscopy has been applied to metabolic studies of prostate cancer in animal models and in humans *(39)*. This technique is particularly attractive as it is possible to obtain metabolic information noninvasively from multiple, distinct regions of prostatic tissue *in situ* and from intact biopsy material. In vitro proton nuclear magnetic resonance (NMR) of extracts of prostatic tissue and cells gives an immediate overview of numerous compounds present in these tissues or cells. We are now able to assign signals in ^{1}H NMR spectra from extracts of the human prostate to spermine. Moreover, in 2D ^{1}H NMR studies of human prostate biopsy tissue, we obtained evidence for the presence of large amounts of spermine *(38)*.

Recently, we explored the applicability of a newly developed NMR technology (i.e., high-resolution magical angle spinning [HRMAS]) to investigate intact ex vivo prostatic tissue material *(40)*. These studies show that with HRMAS a far higher sensitivity and resolution can be achieved as compared with conventional ex vivo NMR methods. The high sensitivity of HRMAS even allows the detection of polyamines and other metabolites in fine needle biopsy material in a relatively short measuring time, enlarging the clinical potential of magnetic resonance spectroscopy. Moreover, improved analysis by adding J-resolved NMR becomes feasible in routine use since this analysis requires only a little time.

Biochemical and NMR studies of polyamine levels in biopsies from prostates of patients showed that normal and benign hyperplastic prostatic tissues have a high spermine content, whereas in tumor tissue, especially in prostatic carcinoma with metastases, spermine levels were reduced *(38–40)*.

Interestingly, the NMR studies show that spermine reduction is accompanied by a reduction in the levels of another abundant metabolite in prostatic fluid, namely citrate. Both spermine and citrate are produced in the epithelial tissue and excreted in the lumen of the prostate, where they appear to form a complex *(11)*.

It is not yet clear whether the reduction of spermine levels is actively involved in the process of prostate carcinogenesis. During prostatic cancer growth the epithelial tissue structure is slowly destroyed, accompanied by a reduction of the luminal space. Hence the reduction of spermine (and citrate) could merely reflect the changes in ductal morphology as a result of a conversion of prostatic tissue from a benign to a malignant phenotype. On the other hand, studies of Zetter et al. *(41–43)* suggest that spermine is actively involved in the regulation of prostate cancer cell growth. High spermine levels present within the prostatic microenvironment suppress prostatic growth by upregulation of AZ. Escape from this regulation may emerge from altered inducibility of AZ

and/or an acquired insensitivity towards spermine. Koike et al. *(42)* reported that rat prostatic cancer cell lines showed a differential sensitivity toward spermine. In the spermine-insensitive cells, AZ levels were not upregulated. AZ possibly acts as an important tumor suppressor by degrading growth-promoting factors, such as ODC and, as was shown recently, cyclin D1 *(43)*.

5. Polyamine Metabolism as a Target for Treatment of Prostate Cancer

Depletion of intracellular polyamines invariably results in growth inhibition or induction of cell death. Therefore, the polyamine metabolic pathway is an attractive target for chemotherapy of prostate cancer.

As shown by our group and others, polyamine inhibitors and polyamine analogs are effective inhibitors of different phenotypes of human prostatic cancer cells and, furthermore, are able to inhibit the in vivo growth of prostatic tumors (reviewed in *44*). Compared with polyamine inhibitors, polyamine analogs may have more clinical value because these compounds can be cytotoxic.

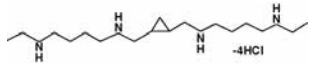
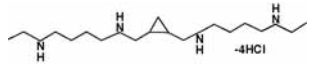
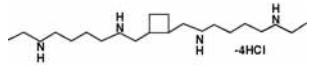
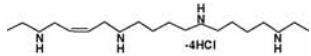
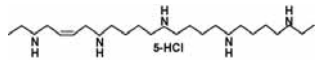
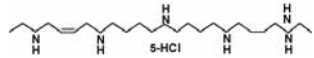
Unfortunately, only a limited number of (biologically different) human prostate cancer cell lines are available. Widely used cell lines, such as DU-145 and PC-3, though undisputed with respect to their origin, lack typical prostate-specific characteristics such as PSA secretion. In contrast, the PC-346C cell line has retained several properties that are characteristic for prostate (cancer) cells, including androgen responsiveness and production of prostate-specific markers *(45)*. PC-346C should therefore be considered a better representative for prostate cancer than the previously studied cell lines. An additional possibility offered by the PC-346C cell line is the option to examine the effect of androgens on the response to polyamine analog treatment.

We have recently tested newly developed polyamine analogs on the in vitro growth of the PC-346C cell line. These compounds are based on the tetramine N^1, N^{12} *bis*(ethyl) spermine (BE-4-4-4) and pentamine 1, 19-*bis*(ethylamine)-5,10, 15-triazonadecane (BE-4-4-4-4), but are restricted in their chain flexibility (developed and manufactured by Prof. Dr. L. J. Marton, Dr. H. Basu, and Dr. B. Frydman, SLIL Biomed Inc., Madison, WI) *(46–49)*. All six polyamine analogs tested had a similar dose-dependent inhibitory effect on the growth of the cell lines DU-145, PC-3, LNCaP, and PC-346C, with activities in the micromolar range (Table 2). The inhibition of growth was from an increased rate of cell death rate because an increase of the proportion of apoptotic cells was seen after polyamine analog treatment *(52)*.

PC-346C cells produce and secrete relatively high levels of PSA in a hormone-dependent fashion. Treatment of PC-346C cultures with polyamine analogs dose dependently suppressed the PSA concentration in culture medium *(52)*.

The growth inhibitory effects of polyamine analogs are likely to depend on the intracellular accumulation of the analog or on the consequent ability to alter the pools of the natural polyamines. Intracellular concentrations of putrescine, spermidine, and spermine, as well as of SL-11098, were determined in PC-346C cells after exposure to the analog for 5 d (Table 3). The results indicated that treatment of PC-346C cells with SL-11098 resulted in a marked decrease of intracellular putrescine, an incomplete reduction of spermidine levels, and increased spermine concentrations. Notably, the

Table 2
Growth Inhibitory Effects of Polyamine Analogs on Human Prostate Cancer Cells

Analog	Structure	IC50-values (μM) DU-145	PC-3	LNCaP	PC-346C
SL-11093		0.26	0.31	2.1	5.6
SL-11098		0.28	0.20	3.5	5.0
SL-11199		0.27	0.19	3.2	5.2
SL-11118		0.25	0.25	2.1	7.6
SL-11121		0.19	0.12	0.9	4.5
SL-11128		0.14	0.09	1.3	6.5

IC_{50} values, drug concentrations required for 50% reduction of cell growth as compared with untreated controls, were determined from dose-effect curves. Results are the mean of at least two different experiments. Variability between duplicate determinations was within 10% for the data shown here.

Table 3
Intracellular Concentrations of Polyamines and SL-11098 in PC-346C Cells Exposed for 5 Days to 5 μM SL-11098 in the Absence and Presence of Androgen (R1881)

Additions	nmol/mg Protein Put	Spd	Spm	SL-11098
None	14.2 ± 8.0	9.1 ± 5.0	11 ± 1.1	ND
10 μM SL-11098	1.1 ± 0.2	5.3 ± 5.0	15 ± 2.0	23.4 ± 2.7
0.1 nM R1881	17.8 ± 1.3	8.7 ± 8	15.5 ± 2.3	ND
0.1 nM R1881 + 10 μM SL-11098	7.4 ± 1.6	7 ± 3.2	31.8 ± 14.8	5.2 ± 2.0

Values, reported in nmol polyamine/mg total cell protein, are the means ± SD from triplicate determinations.

ND, not detectable.

accumulation of SL-11098 in the cells was four to five times higher in the absence of androgen (Table 3). The levels of all three polyamines in SL-11 098–treated cells were lower in the absence than in the presence of androgen.

Polyamine analogs have been shown to be effective inducers of AZ *(50,51)*. Our recent studies using GFP-conjugated ODC *(20)* showed that treatment of PC-346C cells with the polyamine analog SL-11093 resulted in an induction of AZ in the nucleus, similar to the effects of putrescine stimulation. AZ colocalized with ODC-GFP also in PC-346C nuclei. These preliminary data indicate that, at least partly, polyamine analogs may act on cell growth by inducing AZ.

6. Conclusions

The studies reviewed here clearly show that the polyamines are of essential importance for the regulation of cell proliferation and differentiation of prostatic glandular epithelial cells. There is also evidence that ODC and polyamines are crucial for cellular transformation of prostatic cells. Our results obtained with xenograft models of prostate cancer indicate that the expression of ODC is higher in the more malignant variants.

Our studies and data from the literature show that changes in the activity of ODC and other polyamine metabolic enzymes and levels of polyamines are accompanied by alterations in cell kinetic behavior (cell growth, apoptosis) of prostatic (cancer) cells. In particular, spermine, present in high concentration in the prostate, seems to inhibit growth of prostatic cancer cells as long as they remain within the confines of the prostate gland. This explains why during the initial phase of the disease prostate cancer generally grows slowly. Conversion of prostatic epithelium into carcinoma is accompanied by a decrease of spermine concentrations. From these data, we infer that disturbance of the ODC/polyamine system might be an important determinant of malignant behavior of prostatic cancer cells.

The standard treatment of disseminated prostate cancer (i.e., androgen ablation therapy) is aimed to stimulate apoptosis in prostatic cancer cells. However, in the long term, androgen ablation therapy fails because of hormone resistance and concomitant escape of androgen ablation-induced apoptosis. Research shows that polyamine analogs are effective inhibitors of growth of prostatic cancer cells. Our results show that these six novel polyamine analogs, some of which are entering clinical trials, are potent inhibitors of prostate cancer cell growth in vitro. Our findings indicate that androgen-depleted, hormone-responsive, and androgen-independent prostate cancer cells have retained, or even gained, sensitivity to polyamine analogs. Therefore, combined therapy by hormone ablation and polyamine analog treatment might be considered as a potential new strategy for the treatment of advanced prostate cancer.

Data concerning gene activity related to ODC/polyamine metabolism during tumor progression of prostate carcinoma are hardly available. Information on which genes are activated or switched off during exposure of prostatic carcinoma cells to drugs interfering with components of the ODC/polyamine system would greatly improve our understanding of the roles of polyamines in prostate physiology.

In conclusion, polyamines are critically involved in the regulation of prostatic (cancer) growth and therefore might be suitable targets for the diagnosis, prognosis, and chemotherapeutic intervention of prostate cancer.

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References

1. Fjosne, H. E., Ostensen, M. A., Haarstad, H., and Sunde, A. (1990) Androgen regulation of polyamine synthesis in seminal vesicle and in different lobes of the rat prostate. *Prostate* **17,** 1–11.
2. Harrison, G. A. (1931) Spermine in human testis. *Biochem. J.* **25,** 1885–1892.
3. Pegg, A. E., Lockwood, D. H., and Williams, A. H. (1970) Concentrations of putrescine and polyamines and their enzymic synthesis during androgen-induced prostatic growth. *Biochem. J.* **117,** 17–31.
4. Fjosne, H. E., Strand, H., and Sunde, A. (1992) Dose-dependent induction of ornithine decarboxylase and S-adenosyl-methionine decarboxylase activity by testosterone in the accessory sex organs of male rats. *Prostate* **21,** 239–245.
5. Cyriac, J., Haleem, R., Cai, X., and Wang, Z. (2002) Androgen regulation of spermidine synthase expression in the rat prostate. *Prostate* **50,** 252–261.
6. Crozat, A., Palvimo, J. J., Julkunen, M., and Janne, O. A. (1992) Comparison of androgen regulation of ornithine decarboxylase and S-adenosylmethionine decarboxylase gene expression in rodent kidney and accessory sex organs. *Endocrinology* **130,** 1131–1144.
7. Betts, A. M., Waite, I., Neal, D. E., and Robson, C. N. (1997) Androgen regulation of ornithine decarboxylase in human prostatic cells identified using differential display. *FEBS Lett.* **405,** 328–332.
8. Torring, N., Vinter, J. .L, Pedersen, S. B., Sorensen, F. B., Flyvbjerg, A., and Nexo, E. (1997) Systemic administration of insulin-like growth factor I (IGF-I) causes growth of the rat prostate. *J. Urol.* **158,** 222–227.
9. Takyi, E. E. K., Fuller, D. J. M., Donaldson, M. J., and Thomas, G. H. (1997) Deoxyribonucleic acid and polyamine synthesis in rat ventral prostrate. Effects of age of the intact rat and androgen stimulation of the castrated rat with testosterone, 5 alpha-dihydrotestosterone and 5 alpha-androstane-3 beta, 17 beta-diol. *Biochem. J.* **162,** 87–97.
10. Chang, C. S., Saltzman, A. G., Hiipakka, R. A., Huang, I. Y., and Liao, S. S. (1987) Prostatic spermine-binding protein. Cloning and nucleotide sequence of cDNA, amino acid sequence, and androgenic control of mRNA level. *J. Biol. Chem.* **262,** 2826–2831.
11. Goueli, S. A., Davis, A. T., Hiipakka, R. A., Liao, S., and Ahmed, K. (1985) Polyamine-stimulated phosphorylation of prostatic spermine-binding protein is mediated only by cyclic AMP-independent protein kinases. *Biochem. J.* **230,** 293–302.
12. Lynch, M. J. and Nicholson, J. K. (1997) Proton MRS of human prostatic fluid: correlations between citrate, spermine, and myo-inositol levels and changes with disease. *Prostate* **30,** 248–255.
13. Rubinstein, S. and Breitbart, H. (1994) Cellular localization of polyamines: cytochemical and ultrastructural methods providing new clues to polyamine function in ram spermatozoa. *Biol. Cell* **81,** 177–183.
14. Hakovirta, H., Keiski, A., Toppari, J., et al. (1993) Polyamines and regulation of spermatogenesis: selective stimulation of late spermatogonia in transgenic mice overexpressing the human ornithine decarboxylase gene. *Mol. Endocrinol.* **7,** 1430–1436.
15. Romijn, J. C. (1990) Polyamines and transglutaminase actions. *Andrologia* **22,** 83–91.
16. Srinath, P., McQuarrie, S. A., and Suresh, M. R. (2002) Comparative uptake of polyamines by prostate and non-prostate cancer cell lines. *Nucl. Med. Biol.* **29,** 497–503.
17. Mi, Z., Kramer, D. L., Miller, J. T., Bergeron, R. J., Bernacki, R., and Porter, C. W. (1998) Human prostatic carcinoma cell lines display altered regulation of polyamine transport in response to polyamine analogs and inhibitors. *Prostate* **34,** 51–60.

18. Schipper, R. G., Penning, L. C., and Verhofstad, A. A. J. (2000) Review: involvement of polyamines in apoptosis. Facts and controversies: effectors or protectors? *Semin. Cancer Biol.* **10,** 55–68.
19. Schipper, R. G. and Verhofstad, A. A. J. (2004) On the role of polyamines in apoptosis, in *COST 922: Biogenically Active Amines in Food, Vol. I* (Wallace, H. M. and Hughes, A., eds.), European Commission, Luxembourg, pp. 101–109.
20. Schipper, R. G., Cuijpers, V. M. J. I., de Groot, L., Thio, M., and Verhofstad, A. A. J. (2004) Intracellular localization of ornithine decarboxylase and its regulatory protein antizyme-1. *J. Histochem. Cytochem.* **52,** 1259–1266.
21. Schipper, R. G., Otten, A. A., Rutten, R. G. J., Lange, W. P., Schalken, J. A., and Verhofstad, A. A. J. (1995) Polyamine metabolism and programmed cell death in prostatic cancer cells. *Urol. Res.* **23,** 249.
22. Rhodes, D. R., Barrette, T. R., Rubin, M. A., Ghosh, D., and Chinnaiyan, A. M. (2002) Meta-analysis of microarrays: interstudy validation of gene expression profiles reveals pathway dysregulation in prostate cancer. *Cancer Res.* **62,** 4427–4433.
23. Schipper, R. G., Romain, N., Otten, A. A., Tan, J., Lange, W. P., and Verhofstad, A. A. J. (1999) Immunocytochemical detection of ornithine decarboxylase. *J. Histochem. Cytochem.* **47,** 1395–1404.
24. Schipper, R. G., Deli, G., Deloyer, P., Lange, W. P., Schalken, J. A., and Verhofstad, A. A. J. (2000) Antitumor activity of the polyamine analog N(1), N(11)-diethylnorspermine against human prostate carcinoma cells. *Prostate* **44,** 313–321.
25. Heston, W. D., Kadmon, D., Lazan, D. W., and Fair, W. R. (1982) Copenhagen rat prostatic tumor ornithine decarboxylase activity (ODC) and the effect of the ODC inhibitor alpha-difluoromethylornithine. *Prostate* **3,** 383–389.
26. Mohan, R. R., Challa, A., Gupta, S., et al. (1999) Overexpression of ornithine decarboxylase in prostate cancer and prostatie fluid in humans. *Clin. Cancer Res.* **5,** 143–147.
27. Bettuzzi, S., Davalli, P., Astancolle, S., et al. (2000) Tumor progression is accompanied by significant changes in the levels of expression of polyamine metabolism regulatory genes and clusterin (sulfated glycoprotein 2) in human prostate cancer specimens. *Cancer Res.* **60,** 28–34.
28. Brausi, M., Bettuzzi, S., Castagnetti, G., Astancolle, S., and Corti, A. (2004) Gene expression analysis in combination with clinical parameters is effective in predicting relapse after radical prostatectomy. *Eur. Urol. Suppl.* **3,** 58–62.
29. Cipolla, B., Guille, F., Moulinoux, J. P., et al. (1994) Erythrocyte polyamines and prognosis in stage D2 prostatic carcinoma patients. *J. Urol.* **151,** 629–633.
30. Moulinoux, J. P., Quemener, V., Delcros, J. G., and Cipolla, B. (1996) Circulating polyamines as biological markers for cancer. In: *Polyamines in Cancer: Basic Mechanisms and Clinical Approaches* (Nishioka, K., ed.), RG Landis, Austin, TX, pp. 233–249.
31. Clark, R. B. and Fair, W. R. (1975) The selective in vivo incorporation and metabolism of radioactive putrescine in the adult male rat. *J. Nucl. Med.* **16,** 337–342.
32. Heston, W. D., Kadmon, D., Covey, D. F., and Fair, W. R. (1984) Differential effect of alpha-difluoromethylornithine on the in vivo uptake of ^{14}C-labeled polyamines and methylglyoxal bis(guanylhydrazone) by a rat prostate-derived tumor. *Cancer Res.* **44,** 1034–1040.
33. Heston, W. D. and Kadmon, D. (1986) alpha-Difluoromethylornithine enhancement of ^{14}C-putrescine uptake by an androgen-dependent prostatic tumor. *J. Urol.* **136,** 944–948.
34. Hwang, D. R., Lang, L. X., Mathias, C. J., Kadmon, D., and Welch, M. J. N-3-[^{18}F]fluoropropylputrescine as potential PET imaging agent for prostate and prostate derived tumors. *J. Nucl. Med.* **30,** 1205–1210.

35. Hwang, D. R., Mathias, C. J., Welch, M. J., McGuire, A. H., and Kadmon, D. (1990) Imaging prostate derived tumors with PET and N-(3-[^{18}F]fluoropropyl)putrescine. *Int. J. Radiol. Appl. Instrum. B* **17,** 525–532.
36. Hiesiger, E. M., Fowler, J. S., Logan, J., et al. (1992) Is [1–^{11}C]putrescine useful as a brain tumor marker? *J. Nucl. Med.* **33,** 192–200.
37. Wang, G. J., Volkow, N. D., Wolf, A. P., et al. (1994) Positron emission tomography study of human prostatic adenocarcinoma using carbon-11 putrescine. *Nucl. Med. Biol.* **21,** 77–82.
38. Van der Graaf, M., Schipper, R. G., Oosterhof, G. O. N., Schalken, J. A., Verhofstad, A. A. J., and Heerschap, A. (2000) Proton MRS of prostatic tissue focused on the identification of spermine: a possible biomarker of malignant behavior in prostate cancer. *Magn. Res. Materials Biol. Phys. Med. (MAGMA)* **10,** 153–159.
39. Kurhanewicz, J., Swanson, M. G., Nelson, S. J., and Vigneron, D. B. (2002) Combined magnetic resonance imaging and spectroscopic imaging approach to molecular imaging of prostate cancer. *J. Magn. Reson. Imaging* **16,** 451–463.
40. Soede-Huijbregts, C., Cuijpers, V., Sedelaar, M., de la Rosette, J., Verhofstad, A., and Heerschap, A. Metabolic analysis of fine needle biopsies of the prostate by high resolution magic angle spinning magnetic resonance spectroscopy. Submitted.
41. Smith, R. C., Litwin, M. S., Lu, Y., and Zetter, B. R. (1995) Identification of an endogenous inhibitor of prostatic carcinoma cell growth. *Nat. Med.* **1,** 1040–1045.
42. Koike, C., Chao, D. T., and Zetter, B. R. (1999) Sensitivity to polyamine-induced growth arrest correlates with antizyme induction in prostate carcinoma cells. *Cancer Res.* **59,** 6109–6112.
43. Newman, R. M., Mobascher, A., Mangold, U., et al. (2004) Antizyme targets cyclin D1 for degradation. A novel mechanism for cell growth repression. *Biol. Chem.* **279,** 41,504–41,511.
44. Schipper, R. G., Romijn, J. C., Cuijpers, V. M. J. I., Verhofstad, A. A. J. (2003) Polyamines and prostatic cancer. *Biochem. Soc. Trans.* **31,** 375–380.
45. van-Weerden, W. M., de-Ridder, C. M., Verdaasdonk, C. L., et al. (1996) Development of seven new human prostate tumor xenograft models and their histopathological characterization. *Am. J. Pathol.* **149,** 1055–1062.
46. Valasinas, A., Sarkar, A., Reddy, V. K., Marton, L. J., Basu, H. S., and Frydman, B. (2001) Conformationally restricted analogs of 1 N, 14N-bisethylhomospermine (BE-4-4-4): synthesis and growth inhibitory effects on human prostate cancer cells. *J. Med. Chem.* **44,** 390–403.
47. Reddy, V. K., Sarkar, A., Valasinas, A., Marton, L. J., Basu, H. S., and Frydman, B. (2001) Cis-unsaturated analogs of 3,8,13,18,23-pentaazapentacosane (BE-4-4-4-4): synthesis and growth inhibitory effects on human prostate cancer cell lines. *J. Med. Chem.* **44,** 404–417.
48. Frydman, B., Blokhin, A. V., Brummel, S., et al. (2003) Cyclopropane-containing polyamine analogues are efficient growth inhibitors of a human prostate tumor xenograft in nude mice. *J. Med. Chem.* **46,** 4586–4600.
49. Frydman, B., Porter, C. W., Maxuitenko, Y., et al. (2003) A novel polyamine analog (SL-11093) inhibits growth of human prostate tumor xenografts in nude mice. *Cancer Chemother. Pharmacol.* **51,** 488–492.
50. Mitchell, J. L., Leyser, A., Holtorff, M. S., et al. (2002) Antizyme induction by polyamine analogues as a factor of cell growth inhibition. *Biochem. J.* **366,** 663–671.
51. Mitchell, J. L., Simkus, C. L., Thane, T. K., et al. (2004) Antizyme induction mediates feedback limitation of the incorporation of specific polyamine analogues in tissue culture. *Biochem. J.* **384,** 271–279.
52. Romijn, J. C., Schipper, R. G., Cuijpers, V. M. J. I., et al. Effects of novel polyamine analogues on androgen-independent and androgen-responsive human prostate cancer cell lines. Submitted.

10

Polyamines in Kidney Biology

Joseph Satriano

1. Introduction

The function of the kidney is largely considered filtration of the plasma compartment and removal of waste liquids and metabolic end products. Of equal importance is the conservation of liquids and solutes by reabsorption that is required to maintain life. The kidney functions to precisely regulate the body's fluid compartment, maintain electrolytic and acid balance of the plasma, and secrete hormones such as renin, erythropoietin, and prostaglandins.

The functional unit of the kidney, the single nephron, simplistically consists of a glomerular capillary network where plasma is filtered into Bowman's capsule followed by the proximal convoluted and straight tubules, the loop of Henle, the distal tubule, and the collecting duct. The glomerulus is composed of a capillary network lined with a thin layer of fenestrated endothelial cells, a glomerular basement membrane, and visceral epithelial cells (podocytes). These elements form the filtering membrane for the blood passing through the glomerular capillary network. Glomerular mesangial cells and the mesangial matrix maintain the structural integrity of the glomerulus and the functional regulation of filtration. The nephron shows a particular organization whereby the end of the thick ascending limb of the loop of Henle makes contact with the vascular pole of its own glomerulus. This particular tubuloglomerular contact site, termed the juxtaglomerular apparatus, forms a regulatory feedback loop. The functional relevance is related to the large amounts of fluid and electrolytes that are filtered from glomeruli into the tubular system, namely about 180 L of fluid and 25 moles of sodium every day in healthy adults. On a standard Western diet, only about 0.5 to 1% of the fluid or sodium filtered in the glomeruli is actually excreted in the urine, the remaining fluid and electrolytes are reabsorbed back into the blood along the tubular and collecting duct system. This implicates a fine coordination of glomerular filtration and subsequent reabsorption of fluid and electrolytes to adjust the urinary excretion according to bodily needs. The juxtaglomerular apparatus significantly contributes to this coordination through the mechanism of tubuloglomerular feedback (TGF). The TGF mechanism

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refers to a series of events whereby changes in the Na^+, Cl^-, and K^+ concentrations in the tubular fluid are sensed by the macula densa cells at the tubuloglomerular contact site via the Na^+-K^+-$2Cl^-$-cotransporter in its luminal membrane. An increase or decrease in late proximal tubular flow rate, and thus in Na^+, Cl^-, and K^+ delivery at the macula densa, elicits inverse changes in glomerular filtration rate (GFR; an indicator of kidney function). For example, high salt arriving at the macula densa would imply a GFR too high to properly reabsorb salt and consequently signals a decrease in the GFR. As a consequence of TGF, the fluid and electrolyte delivery to the distal nephron is kept within certain limits that facilitate the fine adjustments in reabsorption or excretion in the nephron distal to the juxtaglomerular apparatus under the control of aldosterone and vasopressin.

These mechanisms that balance filtration with excretion and reabsorption are rapidly brought into play on environmental challenge. The reserve of the kidney is large and considerable damage must occur before impaired function is evident. In this chapter, we will discuss such environmental challenges and pathological models of the kidney and the response of polyamines, and where appropriate, arginine and nitric oxide, under these conditions.

Kidney carcinomas will not be discussed here because cancer will be covered much more extensively in other chapters of this book. That said, a recent report on renal cell carcinomas deserves attention. Renal cell carcinomas arise from tubule epithelium and account for 85–90% of all renal cancers in adults. Recently, the antispermine monoclonal antibody, Spm8-2, demonstrated the highest predictive values of all prognostic factors for clinical outcome of renal cell carcinoma patients *(1)*. Spm8-2 could be used at time of radical nephrectomy for defining patients at high risk for progression. Because polyamine levels are typically elevated in neoplastic cells relative to their normal counterparts, this assay may prove to be a valuable prognostic tool for a variety of carcinomas.

2. Localization of Polyamine-Related Enzymes in the Kidney

The basic pathways of polyamine biosynthesis and interconversion are shown in Fig. 1. Ornithine decarboxylase (ODC), the first rate-limiting enzyme of polyamine biosynthesis, is found throughout the kidney. The highest concentration is found in the proximal tubule, followed by the distal and then collecting tubules *(2)*, with activity primarily localized to the proximal tubules *(3–5)*. Expression of ODC, along with ornithine aminotransferase (OAT) is primarily in the outer (convoluted) proximal tubule, whereas arginase II is found in the inner (straight) proximal tubule *(6)*. Because arginase does not appear to specifically colocalize with ODC, ornithine reabsorbed along the proximal convoluted tubule may be the primary source of substrate for ODC *(5)*. Maintenance of plasma arginine levels is a function of biosynthesis in the kidney and dietary intake. Interestingly, arginine synthesis is localized to the proximal convoluted tubule with decreasing activity noted along the proximal straight tubule *(7)*. *S*-adenosylmethionine decarboxylase (AdoMetDC) required for the conversion from lower to higher order polyamines is also found primarily in the cortical tubules, with immunoreactivity strongest at the inner part of the cortex *(8)*. Acetyl CoA:Spd/Spm

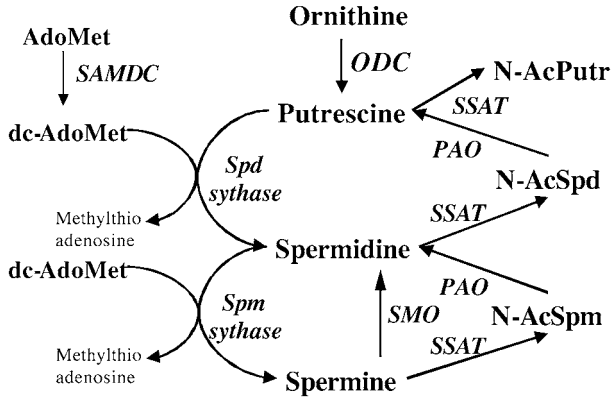

Fig. 1. Polyamine pathways. Ornithine decarboxylase, the first enzyme of polyamine biosynthesis, converts ornithine to putrescine. Enzymes for the forward conversion of polyamines include *S*-adenosylmethionine decarboxylase, spermidine synthase, and spermine synthase. Enzymes involved in back conversion from higher-to-lower order polyamines include acetyl CoA:Spd/Spm *N*-acetyltransferase, polyamine oxidase, and spermine oxidase. AdoMet, *S*-adenosylmethionine; dc-AdoMet, decarboxylated *S*-adenosylmethionine; *N*-Ac, *N*-acetylated; ODC, ornithine decarboxylase; PAO, polyamine oxidase; Putr, putrescine; SAMDC, *S*-adenosylmethionine decarboxylase; SMO, spermine oxidase; Spd, spermidine; Spm, spermine; SSAT, acetyl CoA:Spd/Spm *N*-acetyltransferase.

N^1-acetyltransferase (SSAT), a component of the back conversion of polyamines is localized to the distal tubules, suggesting a differential localization of polyamine synthesis and degradation along the nephron segments *(3)*.

3. Renal Hypertrophy

The kidney has a large reserve and can compensate for stresses, such as the loss of a kidney, with compensatory hypertrophic growth. Experimental models of kidney hypertrophy include unilateral nephrectomy, high-protein diet, and administration of steroids.

3.1. Unilateral Nephrectomy

Unilateral nephrectomy, the surgical removal of one kidney, results in a rapid increase in ODC activity and a gradual increase in kidney weight *(9)*. However, the relevance of ODC induction in this and other models was put into question when inhibition of enzymatic activity with the selective inhibitor 2-(difluoromethyl)-ornithine (DFMO) prevented ODC activity, but not growth. Numerous other apparently contradictory reports in the literature regarding the induction of ODC activity in growth raise two basic discrepancies: (1) ODC is one of the most highly regulated, yet short-lived, eukaryotic enzymes. It is a “rapid”’ response to synthesize polyamines that is responsive to all growth factors. It is a proto-oncogene that is significantly elevated in tumors. However, aside from cellular transformation in which activity is maintained at high levels, ODC activity is typically elevated for hours to days, not weeks. (2) Inhibition of

ODC activity by DFMO for extended periods is generally ineffective. Many of the same factors that induce ODC activity stimulate polyamine transport. However, the onset of *de novo* synthesis of polyamine transporters is slower than activation of ODC, and the duration is longer (i.e., a "sustained" response). Furthermore, DFMO and many other such inhibitors result in a compensatory increase in polyamine transport *(10,11)*, which substitutes for *de novo* polyamine biosynthesis in targeted cells *(12)*. Inhibition of ODC or the polyamine-converting enzymes alone have not fared well in limiting cell proliferation or hypertrophy in in vivo experimental models or clinical trials because of compensatory polyamine transport. Careful analysis, such as by Humphreys et al. in unilateral nephrectomy *(9)*, shows that despite DFMO inhibition of ODC activity, intracellular polyamine levels are largely unaffected after prolonged periods, and, as such, DFMO alone does not prevent compensatory hypertrophy. Seiler demonstrated the importance of compensatory polyamine uptake by evaluating tumor growth in the Lewis lung carcinoma model *(13)*. DFMO treatment did not significantly affect tumor growth, yet growth was completely abolished if DFMO administration was combined with a polyamine-deficient diet. This experiment illustrates the need to address the compensatory uptake of polyamines, in addition to ODC inhibition, in regulating intracellular polyamine levels in animal models.

3.2. High-Protein Diet

Renal mass increases in animals fed a high-protein diet. These animals demonstrate increases in kidney ODC and AdoMetDC activities, as well as GFR *(14–16)*. Conversely, fasting decreases ODC activity *(14)*. A high-protein diet increases the half-life of ODC, which principally accounts for the increase in its expression *(16)*. The mixture of the amino acids glutamate, aspartate, and alanine reproduced the increase in renal ODC activity observed in a high-protein diet *(14)*. In chronically uremic rats, a high-protein diet did not increase AdoMetDC activity *(15)*. ODC activity was not evaluated in that study.

3.3. Steroid-Mediated Hypertrophy

The most studied model of polyamines in kidney growth is androgen-mediated renal hypertrophy. Testosterone administration to castrated mice increases kidney ODC activity more than 1000-fold with a moderate and transient increase in AdoMetDC activity, accompanied by an increase in putrescine and spermidine *(17)*. Androgen administration also increases the half-life of these enzymes *(18)*. The marked conversion of ornithine to putrescine results in a large increase in putrescine excretion into the urine *(19)*, and an increase in diamine oxidase (DAO) activity, which initiates the terminal catabolism of putrescine *(20)*. The rapid increase in putrescine, followed by cellular export, excretion, and catabolism may explain the transient induction of AdoMetDC. Testosterone also increases kidney SSAT messenger RNA (mRNA) expression *(21–23)*. Induction of ODC mRNA localized to the proximal tubule and SSAT mRNA to the distal tubule of castrated mice administered androgens *(21)* could explain why kidney hypertrophy is primarily confined to the proximal tubule. Dudkowska et al. *(22)* observed an increase in SSAT mRNA by testosterone-induced hypertrophy and by drug injury-dependent hyper-

plasia. However, whereas drug-mediated hyperplasia demonstrated an increase in SSAT activity, testosterone-treated animals demonstrated a decrease in activity. The activity of polyamine oxidase (PAO), the other enzyme in the back conversion pathway of polyamines, increases slightly after testosterone treatment in young, 20- to 50-d-old mice, but then decreases in 70-d-old mice *(24)*. The transient, rather than sustained, increase in AdoMetDC, and the increase of putrescine export and putrescine catabolism suggest regulatory effects to compensate for a rapid overproduction of putrescine in response to testosterone. The transient nature of AdoMetDC would imply limited conversion of putrescine to higher order polyamines, and may explain the muted responses by SSAT and PAO.

Testosterone stimulates calcium fluxes and mobilization of intracellular calcium with resultant receptor dependent increases in endocytosis, hexose transport, and amino acid transport in the mouse kidney cortex. DFMO inhibition of ODC blocks these induced effects, implying polyamines act as requisite secondary messengers in this response *(25)*.

Increases in ODC activity and polyamine biosynthesis are common themes in all models of renal hypertrophy.

4. Glomerular Disease

Glomerular injury is the primary cause of kidney disease. The kidney can be the primary organ involved, or one of the many organs damaged by systemic disease. The deposition or formation of antigen–antibody complexes is the principal mechanism of glomerular injury. Immune-mediated glomerular injury is associated with induction of inducible nitric oxide synthase (iNOS) and the production of large amounts of nitric oxide (NO). This has been well documented in a number of renal glomerular diseases, such as lupus nephritis, mesangioproliferative glomerulonephritis, and kidney transplant rejection. To better understand the potential role of polyamines in these and other diseases, we will look at the yin-yang relationship of these arginine-based NO and polyamine pathways.

4.1. Arginine Pathways

The kidney proximal tubule is the principal site of systemic arginine biosynthesis. In addition to its role as a component of protein synthesis, arginine is a precursor for the production of both NO and ornithine. NO has evolved into a unique molecule involved in the regulation of diverse physiological, pathological, pharmacological, and toxicological processes. Certain cytokines and microbial products induce the iNOS isoform of NOS. The high-output production of NO from this isoform can, via interactions with reactive oxygen species, form reactive nitrogen oxide species (RNOS). RNOS formation is likely to occur during conditions of oxidative stress and can lead to lipid peroxidation, DNA damage, oxidation of thiols, and nitration of tyrosine residues *(26)*. Thus, high output of NO evokes protective actions in mammalian tissues to result, in part, from its cytostatic/bactericidal activity toward certain pathogens.

Juxtaposed to the cytostatic effects of NO in pathological conditions is the production of ornithine from arginine, which is the precursor of the pro-proliferative

polyamines. Arginine, but not ornithine, deprivation induces compensatory polyamine transport *(10)*. This observation supports the view that arginine is at the crux of polyamine synthesis, as well as NO generation. OAT also metabolizes ornithine to proline for collagen and extracellular matrix production. The arginine/iNOS/NO and arginine/arginase/ornithine pathways are temporally regulated in acute inflammatory models, such as wound healing and experimental glomerulonephritis *(27–29)*. The production of NO from arginine by iNOS constitutes the early phase response, whereas arginine conversion to ornithine, which is a prerequisite to proliferative polyamine biosynthesis and the generation of proline for extracellular matrix production, comprise the later repair phase responses (Fig. 2). It is easy to see the utility of temporally separating these arginine pathways. However, the mechanisms responsible for this coordination are not well understood.

Another arginine pathway whose existence is debated in mammals is the conversion to agmatine by arginine decarboxylase *(30)*. Recently, the intracellular level of agmatine in the rat kidney was reported to be approx 430 μ*M*, with plasma concentrations of 2.8 μ*M* *(31)*. Agmatine and its metabolites can affect both iNOS-generated NO *(32)* and intracellular polyamine levels required for growth (Fig. 2). Agmatine can induce antizyme (AZ) or SSAT expression to affect intracellular polyamine levels *(33,34)*. High intracellular polyamine concentrations induce AZ expression via a programmed +1 ribosomal frameshift *(35)*. This novel mechanism of induction affords rapid modulation in response to intracellular polyamine concentrations. However, the cell is required to constitutively maintain levels of AZ mRNA requisite for this response, which underscores the importance of this system. In addition to inhibiting polyamine biosynthesis, AZ also suppresses polyamine transporters *(36,37)* and increases polyamine export *(38)*. This unique negative feedback system is effective in limiting intracellular polyamine levels. AZ will be discussed in more detail in other sections of this book. In a process that would complement AZ-mediated polyamine reduction, agmatine can induce SSAT activity. This was shown in rat liver cells *(34)*, Ha-ras transformed NIH/3T3 fibroblast, and MCT and OK proximal tubule cell lines (unpublished data). From these and other data, we hypothesized that agmatine constitutes a gating mechanism that aids in the regulation and transition of the early NO/cytostatic, and later, polyamine/proliferative phases in an acute inflammatory response (for a detailed review, *see* ref. *39*).

4.2. Thy-1 Glomerulonephritis Model

Injection of antibody to a Thy-1-like epitope present on glomerular mesangial cells in rats initiates the anti-thymocyte serum model of mesangial proliferative glomerulonephritis. An inflammatory response predominates the first day after anti-Thy-1 (Thy-1) injection, with high NO production and mesangiolysis. The destruction of glomerular mesangial cells results in a rapid decline in GFR, an indicator of kidney function. By d 2, NO levels decrease, and by d 4 the mesangial cells have nearly repopulated and the GFR practically normalized. The mesangial cells continue to proliferate and, by d 7, the glomeruli are hypercellular with accumulated mesangial matrix, a condition that causes the GRF to again decline. In this “single hit” Thy-1 model, the disease no longer progresses, but rather resolves via apoptotic remodeling *(40)*.

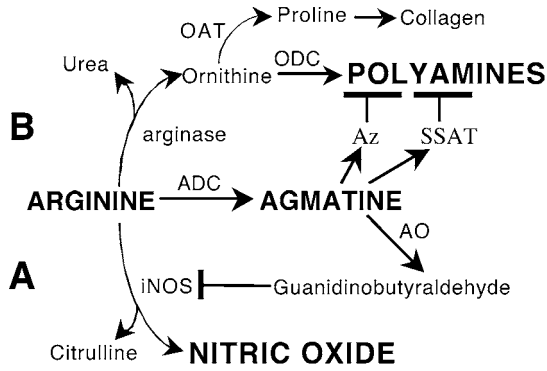

Fig. 2. Arginine pathways. In acute inflammatory models, **(A)** arginine to nitric oxide (NO) production is an early phase response. NO is an antiproliferative molecule resulting, to some degree, from nitrosylation and inactivation of ornithine decarboxylase (ODC). **(B)** The production of ornithine and subsequent metabolism to the pro-proliferative polyamines (ODC) and to proline (OAT) for extracellular matrix production is a later repair phase response. Agmatine induction of antizyme and SSAT can aid in the regulation of intracellular polyamine levels in normal cell homeostasis, in part by adding to the free polyamine pool. Induction of amine oxidase metabolizes agmatine to a moiety that selectively inhibits inducible nitric oxide synthase (iNOS). Suppression of iNOS also occurs by several other factors released during inflammation, such as cytokines. Besides suppressing iNOS, the metabolism of agmatine would shift its repression away from the polyamines and be permissive for growth in the repair phase. Bars represent negative regulation. ADC, arginine decarboxylase; AO, amine oxidase; OAT, ornithine aminotransferase; SSAT, acetyl CoA:Spd/Spm *N*-acetyltransferase.

Thy-1-mediated glomerulonephritis affords a model to evaluate experimental perturbations in these arginine pathways. After an early onset of iNOS induction and NO generation, increases in arginase, ODC, and OAT correlate with the repair phase (i.e., cell proliferation and collagen synthesis) of the response *(41)*. Inhibition of NOS by N(G)-monomethyl-L-arginine (L-NMMA) administration in experimental glomerulonephritis increases both the magnitude and onset of the repair phase response *(28)*, indicating competition for substrate by the two enzymes, or interregulation of these two pathways by NO. Competition for arginine by these enzymes is well documented in other models *(42)*. However, in the Thy-1 model, iNOS and arginase activities are observed in different cell types—macrophage and mesangial cells, respectively—thereby effectively limiting competition. NO can directly nitrosylate and inhibit ODC activity *(29)*. Nitrosylation is readily reversible and dependent on the oxidative state of the cell. It becomes more prevalent when oxidative stress compromises intracellular thiol levels, as mentioned previously in Subheading 4.1.

DFMO administration in the Thy-1 model successfully inhibits ODC activity but had no effect on mesangial cell hyperplasia, glomerular matrix protein expression, or albuminuria *(43)*. This may be owing to a compensatory polyamine uptake mechanism. Agmatine was effective in this Thy-1 model in reducing ODC activity, hyperplasia, and

matrix formation and preventing the decline in GFR *(44)*. A direct effect of agmatine administration on iNOS activity was not statistically significant in these experiments, even though the decline in GFR was eliminated during the initial "NO" phase. This was likely the result of the very indirect technique of measuring NO generation ex vivo and should be reevaluated.

4.3. Lupus

Systemic lupus erythematosus (SLE) is an immune disorder characterized by numerous autoantibodies. The kidney is involved in virtually all cases of SLE. It can manifest as mesangial lupus glomerulonephritis, focal proliferative glomerulonephritis, diffuse proliferative glomerulonephritis, or membranous glomerulonephritis. Glomerulonephritis is a major cause of morbidity and mortality in SLE. The MRL lpr/lpr mouse model produces chronic and progressive glomerulonephritis. Administration of arginine in the drinking water of MRL lpr/lpr mice was associated with increased blood and urine NO levels, albuminuria, extracellular matrix accumulation, and mortality, even though anti-ds DNA-immunoglobulin (Ig)A and renal Ig deposition did not change with the treatment *(45)*. In accord with these data, DFMO administration has proven quite effective in this model. MRL lpr/lpr mice administered DFMO demonstrated lower anti-DNA antibodies *(46)*, decreased interstitial inflammation, perivascular inflammation, vasculitis, and glomerulonephritis *(47)*, with a resultant increase in mean survival time *(46–48)*. These findings were verified in a different genetic background in lupus-prone NZB/W mice in which DFMO treatment also decreased anti-DNA antibody by approx 80% with resultant decreases in proteinuria and blood urea nitrogen (BUN) levels *(49)*. ODC activity is elevated in MRL lpr/lpr mice, as expected from the DFMO results, but ODC mRNA is decreased suggesting posttranscriptional regulation of the enzyme *(48)*.

5. Chronic Kidney Disease

Chronic renal failure is the end result of a variety of kidney diseases. It is the major cause of death from renal disease. The route to chronic renal failure advances through several stages demarcated by progressive decreases in GFR. The first stage, diminished renal reserve, denotes a GFR approx 50% of normal. The patients are asymptomatic, with normal serum BUN and creatinine levels, attesting to the large reserve of the kidney before damage affects health. Renal insufficiency is when the GFR is 20 to 50% of normal, with azotemia (an increase in BUN and creatinine levels), polyuria, and nocturia. It is usually associated with anemia and hypertension. Renal failure occurs when the GFR drops below 20–25% of normal. At this stage, the kidneys cannot regulate properly. Chronic kidney disease is associated with edema, metabolic acidosis, and hypocalcemia. The terminal stage of uremia, in which the GFR drops below 5%, is end-stage renal disease.

5.1. Remnant Kidney Model

The remnant kidney model comprises a 5/6 nephrectomy (or more), which institutes renal insufficiency and uremia. There is an increase in single nephron GFR commensurate with the amount of renal mass ablated. This hypertrophic response is considered beneficial as it attempts to minimize the decline in total GFR and renal plasma flow.

Yet, unlike compensatory hypertrophy in the unilateral nephrectomy model, disease is progressive and relentless. It is accompanied by hypertension, proteinuria, and progressive glomerulosclerosis. Endothelial cells show membranous whorls and lifting, epithelial cells show protein reabsorption droplets associated with proteinuria and foot process fusion, and mesangial cells demonstrate expansion with mesangial thickening. A low-protein diet slows the progression of disease parameters and has been attributed to a decrease in hyperfiltration load *(50)*. Contrary to what one might expect from the effects of a low-protein diet, arginine supplementation resulted in higher GFR and renal plasma flow, and reduced glomerular and tubulointerstitial lesions in rats fed a normal diet *(51)*. ODC mRNA and enzyme activity are shown to decrease in the remnant kidney from d 2 on, yet renal polyamine content is unchanged *(52)*. Whether arginine supplementation increased polyamine levels after the initial insult, relative to uremic rats without arginine supplementation to aid in increasing compensatory growth of the remnant kidney, was not evaluated.

5.2. Hypertension

The kidneys play an important role in maintaining blood pressure. Renin produced by the kidneys will form angiotensin II (AII). AII is a vasoconstrictor molecule that also stimulates aldosterone secretion, which increases reabsorption of sodium and water. Increasing peripheral resistance and blood volume affects blood pressure. The kidney is important in maintaining sodium homeostasis. A fall in GFR will increase sodium reabsorption in an attempt to conserve sodium. Finally, the renal vasculature is maintained in a balance of vasoconstrictor (e.g., AII, endothelin) and vasodilator (e.g., NO, prostaglandins, platelet-activating factor) molecules. A shift in the equilibrium of any of these mechanisms can result in the pathogenesis of hypertension. It is not surprising then that hypertension accompanies chronic kidney disease.

Functional upregulation of AII mediates salt-sensitive hypertension in the Dahl salt-sensitive rat model *(53)*. Increased AII can activate nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide phosphate oxidase, which produces superoxide anion (O_2^-). Interaction of O_2^- with NO produces peroxynitrite ($OONO^-$), an RNOS. This could decrease bioactive NO for vasodilation, thus skewing the balance toward vasoconstriction and hypertension. On a high-salt diet, these rats exhibit increased blood pressure, a progressive decline in GFR, proteinuria, and glomerulosclerosis. Supplementation with arginine proved beneficial in alleviating hypertension and renal complications *(54)*. In the Dahl salt-sensitive model, exogenous addition of spermidine attenuated the rise in blood pressure, whereas DFMO exaggerated the hypertension in high salt-treated, salt-sensitive rats *(55)*. In that report, ODC protein expression in the proximal tubules increased, yet enzyme activity decreased in the hypertensive, salt-sensitive animals. The effects of arginine supplementation on enzyme activity and polyamine production were not evaluated. A detailed description of polyamines and hypertension in the lung is presented in another chapter in this book (*see* Chapter 11).

5.3. Chronic Renal Failure

Patients with chronic renal failure display high polyamine levels. These levels are lowered significantly after dialysis *(56,57)*. Polyamines are considered uremic toxins

in renal failure patients. Only recently has this perception been properly investigated. In a careful study by Sakata et al. *(58)*, analysis of the metabolic intermediates of polyamines revealed production of the oxidized product, acrolein, correlated with the degree of renal failure. Increased levels of polyamine oxidase in the blood of patients with chronic renal failure appeared responsible for the increased production of acrolein. Thus, therapeutic intervention with a polyamine oxidase inhibitor holds the hope of delaying disease progression of chronic renal failure and lowering mortality.

6. Acute Renal Failure

Acute renal failure (ARF) signifies rapid and severe suppression of renal function and urine flow. It is a common clinical condition associated with high morbidity and mortality. There are several conditions that can cause ARF, including urinary obstruction, organic vascular obstruction, massive infection, rapidly progressive glomerulonephritis, acute tubulointerstitial nephritis, disseminated intravascular renal coagulation, and acute tubular necrosis (ATN).

6.1. Acute Tubular Necrosis

The most common cause of ARF is ATN. Damage to the renal tubular epithelial cells in ATN results from ischemic or nephrotoxic causes. Tubular damage likely leads to the perturbation of several pathways. It can shift the balance of vasoconstrictors and vasodilators toward vasoconstriction. This is mediated in part by TGF and increased AII. Cast formation in ATN can lead to tubular blockage. In addition, excess back leak into the neighboring interstitium could eventually collapse the damaged tubule. Toxins can also cause lesions in the glomerular capillary wall and alter the ultrafiltration coefficient. All of these disturbances will decrease the GFR and result in oliguria. Progression is rapid and leads to high salt and water retention, azotemia, metabolic acidosis, and hyperkalemia; in other words, persistent renal failure. Fortunately, ATN is reversible. The tubule epithelial cells begin to repopulate at the onset of recovery, but the integrity of the tubules remain compromised so fluid and electrolyte loss occurs. Because of this loss, hypokalemia becomes a clinical issue. Prognosis of ATN depends on the cause, the stage of disease progression, the involvement of other organs, and appropriate management of blood electrolytes and water balance.

Arginine supplementation provides beneficial effects in the renal ischemia reperfusion injury (IRI) and nephrotoxic models of ARF, at least in part, the result of increases in NO generation *(59)*. Until recently, little has been described about polyamine regulation in these renal models. Zahedi et al. *(60)* reported renal IRI significantly increased SSAT in renal tubules, with consequent increases in putrescine levels resulting from back conversion of polyamines. The authors went on to show that SSAT induction was specific to tubular injury as *cis*-platinum-mediated tubular injury increased SSAT protein expression, but uremia without tubular injury promoted by sodium depletion did not. An increase in back conversion of polyamines would generate H_2O_2 as a metabolic byproduct that could damage the tubules. This was observed in cultured kidney cells overexpressing SSAT *(61)*. Furthermore, SSAT protein expression was upregulated by 2 h and was maintained over the 48-h experimental period. A characterized marker of

renal cell injury, kidney injury molecule-1 (KIM-1), was transiently elevated by 12 h with maximal expression at 24 h after IRI. These data suggest SSAT may be an early marker of acute tubule injury and pathogenesis to ARF. Such a diagnostic marker could be decisive in early clinical detection and intervention.

7. Diabetes

Diabetes mellitus results in a multifactorial etiology. Type 1 diabetes affects the kidney in stages. At the very onset of diabetes, the kidney grows large and GFR becomes supranormal. Sclerosis and kidney failure occur many years later. The contemporary management of patients with diabetes is aimed toward slowing the progression to kidney failure after the onset of proteinuria and sclerosis. However, even the strictest regiment cannot entirely eradicate disease progression. The mechanisms responsible for the onset and progression of this disease have not been fully resolved.

7.1. Tubular Hypothesis

Many explanations have been offered for the elevated GFR and hyperfiltration in early diabetes. Most of these explanations are now cited in standard reference books and relate to signaling abnormalities within the glomerular microvessels (i.e., that the onset of renal diabetic complications are of glomerular origin). A recent approach argues that diabetic glomerular hyperfiltration is rooted, at least in part, in events that occur in the proximal tubule. There is now convincing evidence for a primary increase of fluid and electrolyte reabsorption in the proximal tubule in rats with streptozotocin (STZ)-induced experimental type 1 diabetes *(62)* and in early type 1 diabetes in humans *(63)*. This rise in reabsorption is primary because it is not the consequence of glomerular hyperfiltration resulting from glomerulotubular balance. The increase in tubular transport in early diabetes mellitus is the combined result of an increased Na^+/glucose cotransport, of tubular growth, and tubular dysfunction *(62)*. Thus, aberrant reabsorption of salts from the proximal tubule results in lower salt concentrations reaching the macula densa. As mentioned, the macula densa is sensitive to salt load and feedback regulates GFR via TGF. Low salt arriving at the macula densa signals an increase in GFR. The spiraling increases in GFR to normalize the salt load at the macula densa lead to glomerular hyperfiltration and eventual glomerular damage (Fig. 3).

7.2. The Growth Response

It is postulated that the increase in size of the proximal tubule would cause an increase in salt reabsorption. But how much does growth alone contribute to the rise in GFR? Pharmacological inhibition of ODC with DFMO to reduce kidney growth directly correlates with reductions in proximal tubular hyperreabsorption in rats with early STZ diabetes *(62)*. This illustrates the impact of tubular growth on kidney function in response to STZ. Before these experiments, proximal tubule hypertrophy was widely accepted to be a compensatory response to the load imposed by hyperfiltration. Understanding that diabetic proximal tubule growth is not just a response to hyperfiltration, but itself affects GFR, allows a better understanding of the mechanisms initiating the disease process and thus potential therapeutic intervention. Glomerular

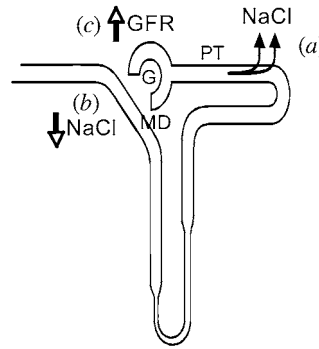

Fig. 3. Proximal tubule-mediated glomerular hyperfiltration. **(A)** Diabetes increases salt reabsorption from the proximal tubule back to the blood. **(B)** This reduces the salt delivered to the macula densa, and **(C)** via tubuloglomerular feedback increases single nephron glomerular filtration rate. This cycle can result in hyperfiltration. G, glomerulus; GFR, glomerular filtration rate; PT, proximal tubule; MD, macula densa; TGF, tubuloglomerular feedback.

expansion would also be affected by suppression of kidney growth. Whether proximal tubule transport is the primary mediator of glomerular hyperfiltration, or whether it is a contributing, yet essential, factor in the negative cycle of progressive dysfunction has yet to be resolved.

Pharmacological inhibition of ODC affects kidney growth and GFR, but is ODC activity pathophysiologically relevant in diabetes? Growth factors associated with the early onset of diabetes, including insulin-like growth factor (IGF-1), hepatocyte growth factor, platelet-derived growth factor, fibroblast growth factor, and synthetic diacylglycerol, all induce ODC activity. Induction of ODC activity can occur via the phosphoinositide 3-kinase (PI3K)/Akt *(64)* and protein kinase C pathways *(65)*. Activation of the PI3K/Akt pathway brings about a complex and coordinated series of events with resultant antiapoptotic, pro-growth, survival, protein translation, cell-cycle entry, and angiogenic effects. IGF-1 may be the most studied mediator of the early growth phase in diabetes, and, as with vascular endothelial growth factor (VEGF), the effects of IGF-1 are attributed principally to the PI3K/Akt pathways. There are numerous examples of IGF-1 administration or overexpression increasing ODC activity, and ODC inhibition suppressing the mitogenic effects of IGF-1 *(66)*. Furthermore, a functional mutation in the IGF-1 receptor prevents IGF-1 induction of ODC activity *(67)*.

STZ-induced diabetes increases ODC activity several fold in the kidney, liver, and intestine, with maximum induction at 24 h. DFMO suppresses hypertrophy and hyperplasia, and in jejunal mucosal enterocytes results in hypoplasia. The rapid yet transient induction of growth factors, such as IGF-1, temporally correlates with ODC expression and activity, induction of intracellular polyamines in the kidney cortex, and the proliferative phase at the onset of STZ diabetes *(62,68)*. ODC inhibition prevents the early increase in 5-bromodeoxyuridine-positive cells and blunts kidney growth, proximal tubular hyperreabsorption, and glomerular hyperfiltration in STZ diabetes *(62)*.

Protein kinase C also gives rise to a set of complex effectors from mitotic to fibrotic, the latter primarily via transforming growth factor (TGF)-β. The diabetic kidney switches from hyperplastic to hypertrophic growth very early in the course of hyperglycemia (e.g., at around d 4 in the STZ model) *(69)*, which matches the time frame of hyperplasia observed using 5-bromodeoxyuridine incorporation *(68)*. TGF-β is an important mediator of this switch in diabetes, with receptor abundance decreasing during the early proliferative phase and increasing during the hypertrophic phase *(69)*. TGF-β arrests cells in the G_1 phase of the cell cycle by induction of the cyclin-dependent kinase inhibitor, $p27^{KIP1}$. Thus kidney growth in the diabetic proximal tubule begins as a mitogen-induced growth response followed by cell-cycle arrest; that is, initial hyperplasia followed by hypertrophy. Renal hypertrophy continued in later stages of diabetes might be more a consequence of decreased proteolysis, thus increased intracellular protein buildup, than cell-cycle arrest *(70)*.

If the early hyperplastic/hypertrophic growth is averted, will the later hypertrophy attributed to decreased proteolytic activity and other "downstream" diabetic complications be diminished as well? There is some evidence that it might *(70)*. Wolf and Ziyadeh *(71)* note: "It is debatable whether or not these hypertrophic processes (in diabetes mellitus) will inevitably lead to irreversible fibrotic changes in humans, but experimental animal models provide ample evidence that this may indeed be the case." In these experimental animal models, growth always precedes glomerulosclerosis. Besides their involvement in the onset of diabetes, polyamines may be involved in the expansion of extracellular matrix and the progression of fibrosis in later stage diabetes as substrates for transglutaminase crosslinking *(72)*. Polyamines and their regulation are emerging as important components in the onset and for the progression of renal complications from diabetes.

8. Conclusions

There are a large number of primary and secondary kidney diseases, and an equally large number of experimental models. Evaluations of the arginine/NOS/NO axis in these models are numerous and growing. Despite the relationship of the arginine/NO and arginine/polyamine pathways in inflammatory diseases, or their requirement in glomerulo or tubulo proliferative and hypertrophic growth, elucidation of polyamines and their regulation in the pathogenesis of renal disease is largely understudied. Considering the success of investigating the influence of polyamines in the examples given in this text, kidney disease appears fertile ground for future polyamine research.

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References

1. Rioux-Leclercq, N., Delcros, J. G., Bansard, J. Y., et al. (2004) Immunohistochemical analysis of tumor polyamines discriminates high-risk patients undergoing nephrectomy for renal cell carcinoma. *Hum. Pathol.* **35,** 1279–1284.

2. Zagon, I. S., McLaughlin, P. J., Seely, J. E., Hoeksema, G. W., and Pegg, A. E. (1984) Autoradiographic localization of ornithine decarboxylase in mouse kidney by use of radiolabeled alpha-difluoromethylornithine. *Cell Tissue Res.* **235,** 371–377.
3. Bettuzzi, S., Marinelli, M., Strocchi, P., Davalli, P., Cevolani, D., and Corti, A. (1995) Different localization of spermidine/spermine N1-acetyltransferase and ornithine decarboxylase transcripts in the rat kidney. *FEBS Lett.* **377,** 321–324.
4. Levillain, O., Diaz, J. J., Blanchard, O., and Dechaud, H. (2005) Testosterone down-regulates ornithine aminotransferase gene and up-regulates arginase ii and ornithine decarboxylase genes for polyamines synthesis in the murine kidney. *Endocrinology* **146,** 950–959.
5. Levillain, O. and Hus-Citharel, A. (1998) Ornithine decarboxylase along the mouse and rat nephron. *Am. J. Physiol.* **274,** F1020–F1028.
6. Yu, H., Yoo, P. K., Aguirre, C. C., et al. (2003) Widespread expression of arginase I in mouse tissues. Biochemical and physiological implications. *J. Histochem. Cytochem.* **51,** 1151–1160.
7. Levillain, O., Hus-Citharel, A., Morel, F., and Bankir, L. (1990) Localization of arginine synthesis along rat nephron. *Am. J. Physiol.* **259,** F916–F923.
8. Gritli-Linde, A., Holm, I., and Linde, A. (1995) Localization of S-adenosylmethionine decarboxylase in murine tissues by immunohistochemistry. *Eur. J. Oral Sci.* **103,** 133–140.
9. Humphreys, M. H., Etheredge, S. B., Lin, S. Y., Ribstein, J., and Marton, L. J. (1988) Renal ornithine decarboxylase activity, polyamines, and compensatory renal hypertrophy in the rat. *Am. J. Physiol.* **255,** F270–F277.
10. Bogle, R. G., Mann, G. E., Pearson, J. D., and Morgan, D. M. (1994) Endothelial polyamine uptake: Selective stimulation by L-arginine deprivation or polyamine depletion. *Am. J. Physiol.* **266,** C776–C783.
11. Satriano, J., Isome, M., Casero, R. A., Jr., Thomson, S. C., and Blantz, R. C. (2001) Polyamine transport system mediates agmatine transport in mammalian cells. *Am. J. Physiol. Cell Physiol.* **281,** C329–C334.
12. Seiler, N. and Dezeure, F. (1990) Polyamine transport in mammalian cells. *Int. J. Biochem.* **22,** 211–218.
13. Seiler, N. (1990) Polyamine metabolism. *Digestion* **46,** 319–330.
14. Sens, D. A., Levine, J. H., and Buse, M. G. (1983) Stimulation of hepatic and renal ornithine decarboxylase activity by selected amino acids. *Metabolism* **32,** 787–792.
15. Skapski, B., Wang, M., Clark, V., and Swendseid, M. E. (1981) Influence of protein intake on S-adenosyl methionine decarboxylase activity in normal and chronically uremic rats. *J. Nutr.* **111,** 1658–1661.
16. Suka, M., Matsufuji, S., and Murakami, Y. (1999) Dietary induction of ornithine decarboxylase in male mouse kidney. *Biochim. Biophys. Acta* **1472,** 455–461.
17. Henningsson, S., Persson, L., and Rosengren, E. (1978) Polyamines and nucleic acids in the mouse kidney induced to growth by testosterone propionate. *Acta Physiol. Scand.* **102,** 385–393.
18. Seely, J. E., Poso, H., and Pegg, A. E. (1982) Effect of androgens on turnover of ornithine decarboxylase in mouse kidney. Studies using labeling of the enzyme by reaction with [14C] alpha-difluoromethylornithine. *J. Biol. Chem.* **257,** 7549–7553.
19. Persson, L. (1981) Putrescine turnover in kidneys of testosterone-treated mice. *Biochim. Biophys. Acta* **674,** 204–211.
20. Pavlov, V. and Dimitrov, O. (2000) Androgenic sensitivity of polyamine-oxidizing enzyme activities in female rat tissues. *Amino Acids* **18,** 399–405.
21. Bettuzzi, S., Strocchi, P., Davalli, P., Marinelli, M., Furci, L., and Corti, A. (2001) Androgen responsiveness and intrarenal localization of transcripts coding for the enzymes of polyamine metabolism in the mouse. *Biochem. Cell. Biol.* **79,** 133–140.

22. Dudkowska, M., Stachurska, A., Grzelakowska-Sztabert, B., and Manteuffel-Cymborowska, M. (2002) Up-regulation of spermidine/spermine N1-acetyltransferase (SSAT) expression is a part of proliferative but not anabolic response of mouse kidney. *Acta Biochim. Pol.* **49,** 969–977.
23. Levillain, O., Greco, A., Diaz, J. J., et al. (2003) Influence of testosterone on regulation of ODC, antizyme, and N1-SSAT gene expression in mouse kidney. *Am. J. Physiol. Renal Physiol.* **285,** F498–F506.
24. Jotova, I., Pavlov, V., Dimitrov, O., and Bachrach, U. (1999) Developmental aspects of polyamine-oxidizing enzyme activities in the mouse kidney. Effects of testosterone. *Amino Acids* **17,** 267–276.
25. Koenig, H., Goldstone, A. D., and Lu, C. Y. (1983) Beta-adrenergic stimulation of Ca2+ fluxes, endocytosis, hexose transport, and amino acid transport in mouse kidney cortex is mediated by polyamine synthesis. *Proc. Natl. Acad. Sci. USA* **80,** 7210–7214.
26. Wink, D. A. and Mitchell, J. B. (1998) Chemical biology of nitric oxide: Insights into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide. *Free Radic. Biol. Med.* **25,** 434–456.
27. Albina, J. E., Mills, C. D., Henry, W. L., Jr., and Caldwell, M. D. (1990) Temporal expression of different pathways of 1-arginine metabolism in healing wounds. *J. Immunol.* **144,** 3877–3880.
28. Cook, H. T., Jansen, A., Lewis, S., et al. (1994) Arginine metabolism in experimental glomerulonephritis: interaction between nitric oxide synthase and arginase. *Am. J. Physiol.* **267,** F646–F653.
29. Satriano, J., Ishizuka, S., Archer, D. C., Blantz, R. C., and Kelly, C. J. (1999) Regulation of intracellular polyamine biosynthesis and transport by NO and cytokines TNF-alpha and IFN-gamma. *Am. J. Physiol.* **276,** C892–C899.
30. Li, G., Regunathan, S., Barrow, C. J., Eshraghi, J., Cooper, R., and Reis, D. J. (1994) Agmatine: An endogenous clonidine-displacing substance in the brain [see comments]. *Science* **263,** 966–969.
31. Lortie, M. J., Ishizuka, S., Schwartz, D., and Blantz, R. C. (2000) Bioactive products of arginine in sepsis: tissue and plasma composition after LPS and iNOS blockade. *Am. J. Physiol. Cell Physiol.* **278,** C1191–C1199.
32. Satriano, J., Schwartz, D., Ishizuka, S., et al. (2001) Suppression of inducible nitric oxide generation by agmatine aldehyde: Beneficial effects in sepsis. *J. Cell Physiol.* **188,** 313–320.
33. Satriano, J., Matsufuji, S., Murakami, Y., et al. (1998) Agmatine suppresses proliferation by frameshift induction of antizyme and attenuation of cellular polyamine levels. *J. Biol. Chem.* **273,** 15,313–15,316.
34. Vargiu, C., Cabella, C., Belliardo, S., Cravanzola, C., Grillo, M. A., and Colombatto, S. (1999) Agmatine modulates polyamine content in hepatocytes by inducing spermidine/spermine acetyltransferase. *Eur. J. Biochem.* **259,** 933–938.
35. Matsufuji, S., Matsufuji, T., Miyazaki, Y., et al. (1995) Autoregulatory frameshifting in decoding mammalian ornithine decarboxylase antizyme. *Cell* **80,** 51–60.
36. Mitchell, J. L., Judd, G. G., Bareyal-Leyser, A., and Ling, S. Y. (1994) Feedback repression of polyamine transport is mediated by antizyme in mammalian tissue-culture cells. *Biochem. J.* **299,** 19–22.
37. Suzuki, T., He, Y., Kashiwagi, K., Murakami, Y., Hayashi, S., and Igarashi, K. (1994) Antizyme protects against abnormal accumulation and toxicity of polyamines in ornithine decarboxylase-overproducing cells. *Proc. Natl. Acad. Sci. USA* **91,** 8930–8934.
38. Sakata, K., Kashiwagi, K., and Igarashi, K. (2000) Properties of a polyamine transporter regulated by antizyme. *Biochem. J.* **347,** 297–303.

39. Satriano, J. (2004) Arginine pathways and the inflammatory response: Interregulation of nitric oxide and polyamines: Review article. *Amino Acids* **26,** 321–329.
40. Savill J. (1999) Regulation of glomerular cell number by apoptosis. *Kidney Int.* **56,** 1216–1222.
41. Ketteler, M., Ikegaya, N., Brees, D. K., Border, W. A., and Noble, N. A. (1996) L-arginine metabolism in immune-mediated glomerulonephritis in the rat. *Am. J. Kidney Dis.* **28,** 878–887.
42. Mori, M., Gotoh, T., Nagasaki, A., Takiguchi, M., and Sonoki, T. (1998) Regulation of the urea cycle enzyme genes in nitric oxide synthesis. *J. Inherit. Metab. Dis.* **21,** 59–71.
43. Ketteler, M., Westenfeld, R., Gawlik, A., de Heer, E., and Distler, A. (2000) Acute glomerular upregulation of ornithine decarboxylase is not essential for mesangial cell proliferation and matrix expansion in anti-Thy-1-nephritis. *Nephrol. Dial. Transplant.* **15,** 16–22.
44. Ishizuka, S., Cunard, R., Poucell-Hatton, S., et al. (2000) Agmatine inhibits cell proliferation and improves renal function in anti-thy-1 glomerulonephritis. *J. Am. Soc. Nephrol.* **11,** 2256–2264.
45. Peters, H., Border, W. A., Ruckert, M., Kramer, S., Neumayer, H. H., and Noble, N. A. (2003) L-arginine supplementation accelerates renal fibrosis and shortens life span in experimental lupus nephritis. *Kidney Int.* **63,** 1382–1392.
46. Thomas, T. J. and Messner, R. P. (1989) Beneficial effects of a polyamine biosynthesis inhibitor on lupus in MRL-lpr/lpr mice. *Clin. Exp. Immunol.* **78,** 239–244.
47. Gunnia, U. B., Amenta, P. S., Seibold, J. R., and Thomas, T. J. (1991) Successful treatment of lupus nephritis in MRL-lpr/lpr mice by inhibiting ornithine decarboxylase. *Kidney Int.* **39,** 882–890.
48. Hsu, H. C., Seibold, J. R., and Thomas, T. J. (1994) Regulation of ornithine decarboxylase in the kidney of autoimmune mice with the lpr gene. *Autoimmunity* **19,** 253–264.
49. Thomas, T. J. and Messner, R. P. (1991) Difluoromethylornithine therapy of female NZB/W mice. *J. Rheumatol.* **18,** 215–222.
50. Hostetter, T. H., Olson, J. L., Rennke, H. G., Venkatachalam, M. A., and Brenner, B. M. (2001) Hyperfiltration in remnant nephrons: a potentially adverse response to renal ablation. *J. Am. Soc. Nephrol.* **12,** 1315–1325.
51. Reyes, A. A., Purkerson, M. L., Karl, I., and Klahr, S. (1992) Dietary supplementation with L-arginine ameliorates the progression of renal disease in rats with subtotal nephrectomy. *Am. J. Kidney Dis.* **20,** 168–176.
52. el-Marjou, A., Bankir, L., Pierrat. D., and Trinh-Trang-Tan, M. M. (1997) Messenger RNA for enzymes of ornithine and polyamine metabolism are selectively underexpressed in kidney of 5/6 nephrectomized rats. *Exp. Nephrol.* **5,** 285–294.
53. Zhou, M. S., Adam, A. G., Jaimes, E. A., and Raij, L. (2003) In salt-sensitive hypertension, increased superoxide production is linked to functional upregulation of angiotensin II. *Hypertension* **42,** 945–951.
54. Chen, P. Y., St John, P. L., Kirk, K. A., Abrahamson, D. R., and Sanders, P. W. (1993) Hypertensive nephrosclerosis in the Dahl/Rapp rat. Initial sites of injury and effect of dietary L-arginine supplementation. *Lab. Invest.* **68,** 174–184.
55. Hayashi, T., Tsujino, T., Iwata, S., et al. (2002) Decreased ornithine decarboxylase activity in the kidneys of Dahl salt-sensitive rats. *Hypertens. Res.* **25,** 787–795.
56. Saito, A., Takagi, T., Chung, T. G., and Ohta, K. (1983) Serum levels of polyamines in patients with chronic renal failure. *Kidney Int. Suppl.* **16,** S234–S237.
57. Takagi, T., Chung, T. G., and Saito, A. (1983) Determination of polyamines in hydrolysates of uremic plasma by high-performance cation-exchange column chromatography. *J. Chromatogr.* **272,** 279–285.

58. Sakata, K., Kashiwagi, K., Sharmin, S., et al. (2003) Increase in putrescine, amine oxidase, and acrolein in plasma of renal failure patients. *Biochem. Biophys. Res. Commun.* **305,** 143–149.
59. Schramm, L., La, M., Heidbreder, E., et al. (2002) L-arginine deficiency and supplementation in experimental acute renal failure and in human kidney transplantation. *Kidney Int.* **61,** 1423–1432.
60. Zahedi, K., Wang, Z., Barone, S., et al. (2003) Expression of SSAT, a novel biomarker of tubular cell damage, increases in kidney ischemia-reperfusion injury. *Am. J. Physiol. Renal Physiol*. **284,** F1046–F1055.
61. Wang, Z., Zahedi, K., Barone, S., et al. (2004) Overexpression of SSAT in kidney cells recapitulates various phenotypic aspects of kidney ischemia-reperfusion injury. *J. Am. Soc. Nephrol.* **15,** 1844–1852.
62. Thomson, S. C., Deng, A., Bao, D., et al. (2001) Ornithine decarboxylase, kidney size, and the tubular hypothesis of glomerular hyperfiltration in experimental diabetes. *J. Clin. Invest.* **107,** 217–224.
63. Hannedouche, T. P., Delgado, A. G., Gnionsahe, D. A., Boitard, C., Lacour, B., and Grunfeld, J. P. (1990) Renal hemodynamics and segmental tubular reabsorption in early type 1 diabetes. *Kidney Int.* **37,** 1126–1133.
64. Shantz, L. M. (2004) Transcriptional and translational control of ornithine decarboxylase during Ras transformation. *Biochem. J.* **377,** 257–264.
65. Jetten, A. M., Ganong, B. R., Vandenbark, G. R., Shirley, J. E., and Bell, R. M. (1985) Role of protein kinase C in diacylglycerol-mediated induction of ornithine decarboxylase and reduction of epidermal growth factor binding. *Proc. Natl. Acad. Sci. USA* **82,** 1941–1945.
66. Olanrewaju, H., Patel, L., and Seidel, E. R. (1992) Trophic action of local intraileal infusion of insulin-like growth factor I: Polyamine dependence. *Am. J. Physiol.* **263,** E282–E286.
67. Kato, H., Faria, T. N., Stannard, B., Roberts, C. T., Jr., and LeRoith, D. (1994) Essential role of tyrosine residues 1131, 1135, and 1136 of the insulin-like growth factor-I (IGF-I) receptor in IGF-I action. *Mol. Endocrinol.* **8,** 40–50.
68. Deng, A., Munger, K. A., Valdivielso, J. M., et al. (2003) Increased expression of ornithine decarboxylase in distal tubules of early diabetic rat kidneys: Are polyamines paracrine hypertrophic factors? *Diabetes* **52,** 1235–1239.
69. Huang, H. C. and Preisig, P. A. (2000) G1 kinases and transforming growth factor-beta signaling are associated with a growth pattern switch in diabetes-induced renal growth. *Kidney Int.* **58,** 162–172.
70. Franch, H. A., Wang, X., Sooparb, S., Brown, N. S., and Du, J. (2002) Phosphatidylinositol 3-kinase activity is required for epidermal growth factor to suppress proteolysis. *J. Am. Soc. Nephrol.* **13,** 903–909.
71. Wolf, G. and Ziyadeh, F. N. (1999) Molecular mechanisms of diabetic renal hypertrophy. *Kidney Int.* **56,** 393–405.
72. Skill, N. J., Johnson, T. S., Coutts, I. G., et al. (2004) Inhibition of transglutaminase activity reduces extracellular matrix accumulation induced by high glucose levels in proximal tubular epithelial cells. *J. Biol. Chem.* **279,** 47,754–47,762.

11

Polyamines in Pulmonary Vascular Biology

Jack W. Olson and Mark N. Gillespie

1. Introduction

Polyamines are essential for cell growth and development. They regulate many functions, including cell division, migration, ion channel regulation, apoptosis, and the cellular synthesis of DNA, RNA, and proteins. A recent review on the roles of polyamines in the lung emphasized studies on respiratory cell biology and polyamine uptake *(1)*. The primary goal of this chapter is to review evidence that polyamines contribute to phenotypical changes in pulmonary vascular cells that underlie the pathogenesis of pulmonary arterial hypertension. Because arginases can regulate polyamines, their potential role in the pathogenesis of pulmonary hypertension and asthma also will be reviewed. The data suggest polyamines may be future therapeutic targets for pulmonary hypertension, although clinical trials measuring polyamines and their regulation are lacking.

2. Overview of Pulmonary Hypertension

Chronic pulmonary arterial hypertension is frequently a fatal disease in humans (reviewed in refs. *2–4*). Its pathobiology is multifactorial and is characterized by progressive narrowing of the small pulmonary arteries. The narrowing is due primarily to vasoconstriction, thrombosis, and most importantly, remodeling of the small pulmonary arteries that causes increased pulmonary vascular resistance and, ultimately, right heart failure. Remodeling occurs in all three layers of the vessel wall and is due in part to proteolysis and the accumulation of extracellular matrix proteins, plus increased cell size and number. Resident vessel wall cells—endothelial, smooth muscle, and fibroblasts—and circulating platelets and inflammatory cells participate in the narrowing process. For example, endothelial cells frequently are injured or dysfunctional. They can display phenotypical abnormalities in proliferation, survival, and neovascularization, and also inappropriate secretion of factors regulating vasodilation, vasoconstriction, growth, and coagulation. Pulmonary arterial smooth muscle cells increase in cell size and number and secrete excessive amounts of extracellular matrix proteins. Mutations or polymorphisms in genes regulating pulmonary arterial smooth muscle or endothelial cell proliferation, apoptosis and differentiation have

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been identified in some patients with inherited or idiopathic pulmonary arterial pulmonary hypertension *(2–4)*.

Although no animal model completely reproduces the pathology of human pulmonary arterial hypertension, the monocrotaline (MCT) and chronic hypoxia rat models mimic several key features and are the most frequently studied (reviewed in refs. *2–4*). Although most studies on the role of polyamines in these rat models have focused on the regulation of polyamines by ornithine decarboxylase (ODC) and polyamine transport, only a few studies have examined the contribution of arginine and arginases.

3. Polyamines and MCT-Induced Pulmonary Hypertension

A single subcutaneous injection of MCT, a plant pyrrolizidine alkaloid, causes pulmonary thrombosis, inflammatory cell infiltration, vasoconstriction, and perivascular edema within 1 to 4 d. Although lung endothelial injury is evident within hours, the progressive vascular remodeling process leading to the development of sustained pulmonary hypertension and right ventricular hypertrophy (RVH) develops over 2–3 wk. The pathology is not reversible and is ultimately fatal. Lung endothelial cell injury and subsequent dysfunctional endothelium are considered the primary events initiating MCT-caused pulmonary hypertension in rats. MCT-injured endothelial cells exhibit altered proliferative, survival, and barrier properties, and they elicit formation of thrombotic lesions, a proinflammatory environment, increased proteolytic activity, altered cellular phenotypes, and extracellular matrix changes. MCT-treated lungs accumulate cytokines, growth factors, and vasoactive peptides. Vascular smooth muscle cells and pericytes respond to MCT by switching to phenotypes that migrate, proliferate, and produce an abnormal extracellular matrix. Vascular cell fate also is altered by increased proteinase activity and subsequent changes in the extracellular matrix.

We reasoned that polyamines may be causally related to these events and, therefore hypothesized that elevated lung contents of polyamines and activities of ODC and *S*-adenosylmethionine decarboxylase (AdoMetDC) would precede development of pulmonary hypertension and RVH. Figure 1 summarizes data showing time-dependent increases in lung ODC and AdoMetDC activities and polyamine contents. Lung ODC activity was increased approximately eightfold on d 1 and remained elevated through d 7 *(5)*. Lung putrescine levels were increased from d 7 through 21, and both spermidine and spermine were first elevated at d 10 after MCT administration *(6)*. This sustained

Fig. 1. (*Opposite page*) Time-dependent changes in lung ornithine decarboxylase (ODC), AdoMetDC, polyamine contents, mean pulmonary artery pressure (Ppa), and right ventricular hypertrophy (RVH) after a single subcutaneous dose of monocrotaline (MCT). RVH was quantitated as the ratio of the right ventricular free wall weight (RV) to the weight of the left ventricle plus septum (LV+S). Controls were given the MCT vehicle (0.9% NaCl) and studied at each time point. Data reported are the fold change from control rats (mean ± the standard error). Control values were: ODC activity = 8.3 ± 1.5 nmoles/total lung weight/60 min; AdoMetDC activity = 2.6 ± 0.25 nmoles/total lung weight/60 min; putrescine = 53.8 ± 5.2 nmoles/total lung weight; spermidine = 930 ± 24 nmoles/total lung weight; spermine = 431 ± 15 nmoles/total lung weight; Ppa = 17.5 ± 0.5 mm Hg; RVH = 0.27 ± 0.005 RV/LV+S. Asterisk (*) indicates data differ from controls at $p < 0.05$. $n = 9$ rats in each group. (Adapted from refs. *6* and *9*.)

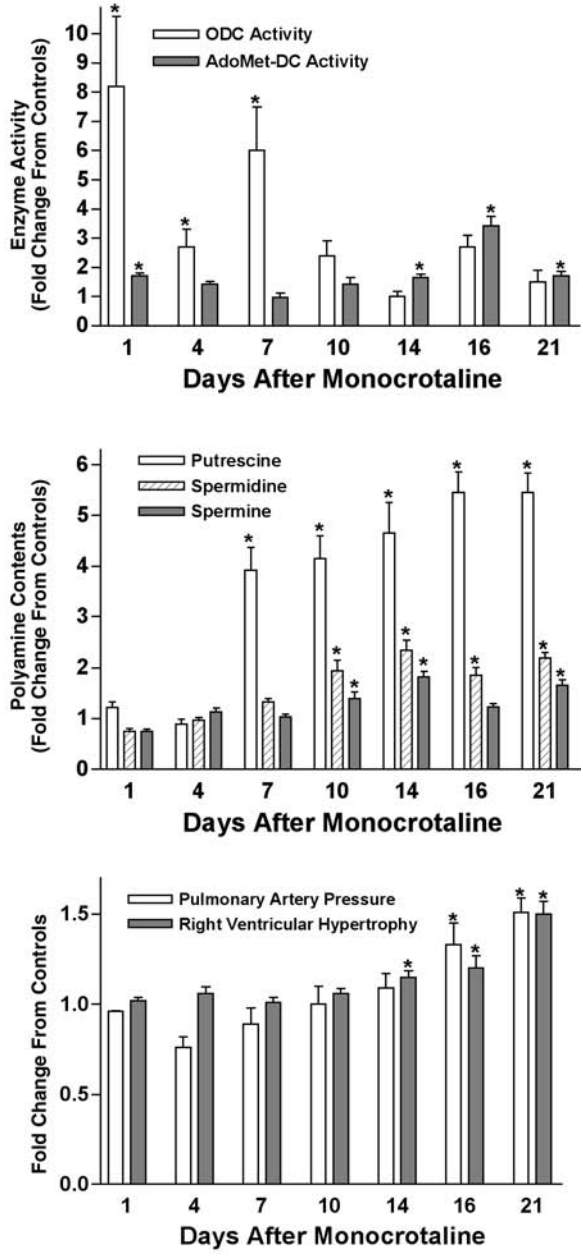
ODC Activity
AdoMet-DC Activity
Enzyme Activity
(Fold Change From Controls)
Days After Monocrotaline
Putrescine
Spermidine
Spermine
Polyamine Contents
(Fold Change From Controls)
Days After Monocrotaline
Pulmonary Artery Pressure
Right Ventricular Hypertrophy
Fold Change From Controls
Days After Monocrotaline

Fig. 1.

elevation of lung polyamine contents and ODC and AdoMetDC activities substantially preceded the development of RVH and pulmonary hypertension, which were first evident at d 14 and 16, respectively *(5,6)*. These changes in lung enzyme activity and contents of polyamines were dependent on the dose of MCT. Furthermore, polyamine catabolism was modulated by MCT *(7)*. Both N^1-acetylspermidine contents and the activity of spermidine/spermine acetyltransferase, an enzyme controlling the back conversion of spermidine and spermine to putrescine, were increased dose- and time-dependently by MCT. Neither N^1-acetylspermine nor N^1-acetylputrescine could be detected in lungs from control rats or from rats treated with MCT. The previously described changes in whole lung contents of polyamines were hypothesized to occur in lung vascular cells because they are primary cellular targets of MCT. As predicted, left pulmonary arterial segments from the first to the sixth intrapulmonary branch had increased contents of putrescine and spermidine at 7 and 21 d after MCT treatment, whereas spermine content was increased at 21 d *(8)*.

If increased ODC activity is necessary for MCT-caused development of pulmonary hypertension, then inhibition of ODC activity should block development of hypertensive vascular disease in MCT-treated rats. Figure 2 shows that continuous treatment with DFMO (α-difluoromethylornithine), a highly specific, enzyme-activated, irreversible inhibitor of ODC activity, prevented the increase in lung putrescine and spermidine contents without significantly altering spermine content *(9,10)*. Importantly, DFMO completely prevented the MCT-increased pulmonary arterial pressure and right ventricle hypertrophy *(6,10)*. The development of lung perivascular edema at d 7 was also blunted by DFMO, whereas the proposed first step of MCT pathology—that is, the hepatic biotransformation of MCT to its active pyrrolic metabolites—was not influenced by DFMO treatment *(9)*. DFMO also prevented the primary causes of MCT-increased pulmonary artery pressure:pulmonary vascular vasoconstriction, thrombosis, and arterial medial thickening *(9,11)* (Fig. 2). Because arterial medial thickening is primarily from increased cell size and number plus accumulation of extracellular matrix proteins, DFMO might target these events. In this context, DFMO prevented MCT-increased DNA synthesis (^{3}H-thymidine incorporation into whole-lung DNA) and thereby may limit vascular cell growth and division *(10)*. DFMO also could prevent MCT-caused alterations in the extracellular matrix, such as the accumulation of fibronectin (FN), elastin, collagen, and tenascin-C *(2)*. FN, which can stimulate multiple signaling events and alter cell fate, is deposited in large amounts in airways, parenchyma, and pulmonary arteries of rats administered MCT *(12)*. In addition, preliminary studies suggest MCT-increased ODC activity may generate FN variants favoring lung remodeling by enhancing rates of *FN* gene transcription and exon-specific alternative splicing *(13)*. DFMO treatment completely prevented the MCT-stimulated splicing of the *FN* IIIA exon and expression of *FN* messenger RNA (mRNA) and FN protein in rat lungs. These results support the concept that elevated ODC activity promotes MCT-induced remodeling of the pulmonary vasculature in part through enhancing *FN* splicing and expression.

Another event contributing to MCT-caused vascular structural changes is pulmonary endothelial cell apoptosis. Preliminary studies have been initiated to examine the roles of ODC in the MCT-caused apoptotic response in rat lungs and cultured rat pulmonary

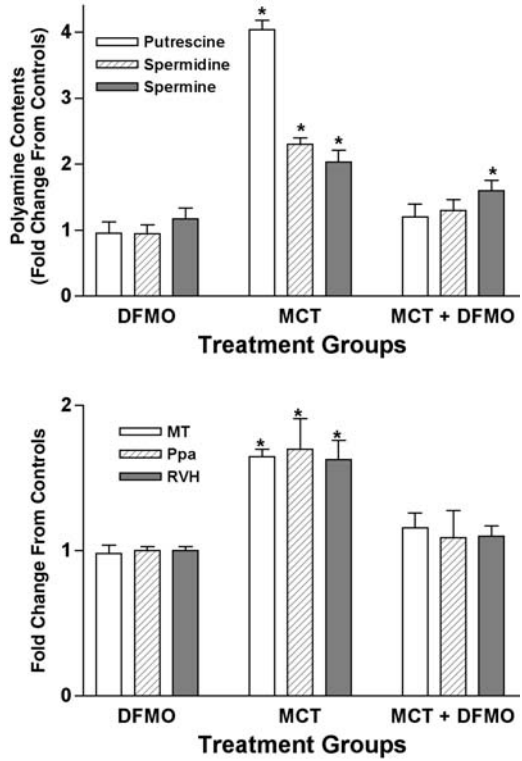

Fig. 2. Continuous DFMO treatment prevents monocrotaline (MCT)-caused changes in rat lung polyamine contents and pulmonary arterial medial thickening (MT) and pressure (Ppa) and right ventricular hypertrophy (RVH). MT was determined in 50- to 100-μm pulmonary arteries as the percent of external diameter (ratio of smooth muscle layer thickness to the diameter between external elastic lamina). Control and DFMO-only rats were given the MCT vehicle (0.9% NaCl) and studied 21 d later. DFMO only and MCT+DFMO rats received 2% DFMO in the drinking water from 4 d before MCT until the study was terminated at 21 d after administration of MCT. Data reported are the fold change from control rats (mean ± the standard error). Control values were: putrescine = 24 ± 9 nmoles/left lung weight; spermidine = 293 ± 47 nmoles/left lung weight; spermine = 154 ± 24 nmoles/left lung weight; MT = 8 ± 0.6% of external diameter; Ppa = 11 ± 2.2 mm Hg; RVH = 0.30 ± .01 RV/LV+S. Asterisk (*) indicates data differ significantly from control or DFMO only groups at $p < 0.05$. $n = 6$ rats in each group. (Adapted from refs. *9* and *15*.)

artery endothelial cells (PAECs) *(14)*. Immunohistochemical analyses revealed that PAECs within the lungs of MCT-treated rats had increased immunostaining for ODC, c-myc, and caspase-3, a key apoptotic enzyme, at d 1, 4, and 7 after MCT administration. DFMO treatment blocked the increase in PAEC caspase-3 immunostaining. Western blot analysis of lungs confirmed that DFMO prevented the MCT-caused activation of caspase 3 and degradation of poly (ADP-ribose) polymerase. In cultured rat PAECs, ODC activity was increased 4, 24, and 72 h after addition of MCT pyrrole, the

active metabolite of MCT. This increase in ODC activity was due in part to c-myc-enhanced ODC transcription. MCT pyrrole also initiated an apoptotic response from 4 to 72 h as indicated by increased annexin-V staining, caspase-3 activity, and DNA laddering. DFMO treatment blocked MCT-increased annexin-V staining and partially blocked and delayed caspase-3 activity and DNA laddering. These data suggest increased ODC activity contributes to MCT-caused PAEC apoptosis and that DFMO targets one of the earliest stages in the development of pulmonary hypertension.

If increased lung contents of polyamines are required for the development of MCT-caused pulmonary hypertension, the protective effects of DFMO should be reversible by elevating lung polyamine contents through supplementation with exogenous polyamines or ornithine. In this regard, the protection afforded by DFMO was reversed by chronic coadministration of ornithine, the substrate for putrescine biosynthesis by ODC *(15)*. The rats receiving the combination treatment of MCT plus DFMO and ornithine had lung polyamine contents at levels normally associated with MCT treatment alone. This confirms that DFMO was acting as a specific inhibitor of polyamine biosynthesis in MCT-induced pulmonary hypertension. Using the same protocol, Hacker *(10)* independently showed ODC and polyamines are essential for the development and progression of MCT-induced pulmonary hypertension.

These experiments provided evidence that continuous DFMO treatment, when initiated at the time of MCT administration, prevents MCT-caused hypertensive vascular disease. These studies did not address whether DFMO can cause regression or prevent progression of the disease after it is established, a question relevant for therapy of humans with pulmonary hypertension. In this context, DFMO treatment, when started at a time well after the early onset of MCT-induced pneumotoxicity, retained at least some of its protective effects *(15)*. DFMO treatment initiated at d 10 after MCT—when lung polyamine contents are elevated, and perivascular edema, inflammatory cell infiltration, vasoconstriction, and vascular remodeling are evident—significantly blunted, but did not completely prevent, the MCT-caused RVH and increases in putrescine and spermidine contents. It thus remains unanswered whether complete regression of MCT-induced pulmonary hypertension can be achieved by treatment regimens that reduce polyamine lung contents to control levels. Perhaps a regimen that combines DFMO plus a polyamine uptake blocker will be required. In this context, MCT can enhance polyamine uptake in cultured vascular cells, although it is not known if lung polyamine transport is altered in MCT-treated rats.

4. Polyamines and Chronic Hypoxia-Induced Pulmonary Hypertension

Although MCT and chronic hypoxia elicit pulmonary hypertension in rats, there are notable differences between these models *(2,3,16)*. Compared with MCT, vascular endothelial injury, and thrombosis, recruitment of circulating leukocytes and perivascular inflammation are less pronounced in hypoxic pulmonary hypertension. In addition, pulmonary vasoconstriction is more significant, type II pneumocyte toxicity is not apparent, and the time to develop pulmonary hypertension is compressed in hypoxic rats. Although it is the cause of death in MCT-treated rats, pulmonary hypertension naturally regresses after hypoxic animals are returned to a normoxic environment.

Because of the differences between the two models, we tested whether the polyamines are mediators of chronic hypoxia-induced pulmonary vascular remodeling.

As with MCT, hypoxia-increased lung polyamine contents temporally preceded development of pulmonary artery hypertension *(17–19)*. Specifically, lung contents of spermidine and spermine increased within 1 d and putrescine within 4 d after continuous exposure to hypoxia (simulated altitude: 4570 m) and these changes occurred before increases in pulmonary arterial pressure and development of RVH (Fig. 3). Unexpectedly, and opposite to MCT-treated rats, lung ODC activity was reduced below control levels from 4 to 14 d of exposure. This response to hypoxia seemed to be specific to ODC activity because hypoxic lungs had sustained increases in AdoMetDC and spermidine/spermine acetyltransferase activities and N^1-acetylspermidine contents. Additional studies tested whether polyamine transport contributed to hypoxia-increased lung polyamine contents. Putrescine transport kinetics were assessed in isolated, salt solution-perfused lungs from rats previously exposed to normoxia or hypoxia for 7 d, a time when ODC was reduced to its lowest level. Accelerated uptake from the vascular compartment plus decreased efflux from the lung contributed to the elevated lung putrescine contents since hypoxia-increased the apparent K_m and capacity for putrescine uptake and the $T_{1/2}$ for loss of putrescine from a slowly effluxing pool. These data support the concept that hypoxia-increased lung polyamine contents are not from ODC activity but instead, result from elevated AdoMetDC and spermidine/spermine acetyltransferase activities coupled with accentuated uptake and decreased efflux of putrescine.

Despite the ability of hypoxia to decrease total lung ODC activity, DFMO attenuated the hypoxia-increased lung contents of putrescine and spermidine, and blunted the increases in pulmonary arterial pressure and medial thickness *(17,18)* (Fig. 4). DFMO did not alter the hypoxia-evoked increase in hematocrit, vasoconstriction, and RVH. These observations suggest that depression of polyamine biosynthesis with DFMO blunts the sustained increase in pulmonary arterial pressure by attenuating hypoxia-induced medial thickening. Although the DFMO-sensitive resident cell types within the rat lung remain unidentified, DFMO may target the recruitment of nonresident lung cells to the pulmonary circulation because these cells likely participate in the hypoxia-caused vascular remodeling process *(16)*.

To begin to identify lung resident cell types in which hypoxia induces polyamine transport in lungs, rat lung and rat main pulmonary arterial explants were incubated with ^{14}C-spermidine in either normoxic or hypoxic environments for 24 h *(20)*. Autoradiographic evaluation revealed ^{14}C-spermidine localized prominently to conduit, muscularized, and partially muscularized pulmonary arteries in hypoxic, but not normoxic, lung tissue and localized to both intimal and medial arterial cells of hypoxic main pulmonary arterial explants, but the increase was most evident in the smooth muscle cells. Experiments using main pulmonary arterial explants denuded of endothelium and in addition, cultured pulmonary artery smooth muscle (PASMCs) cells and PAECs showed that endothelial-derived factors are required for hypoxia-enhanced spermidine uptake in the underlying smooth muscle. Rat PASMCs cultured in normoxic environment were found to express two discrete transporter systems: one for

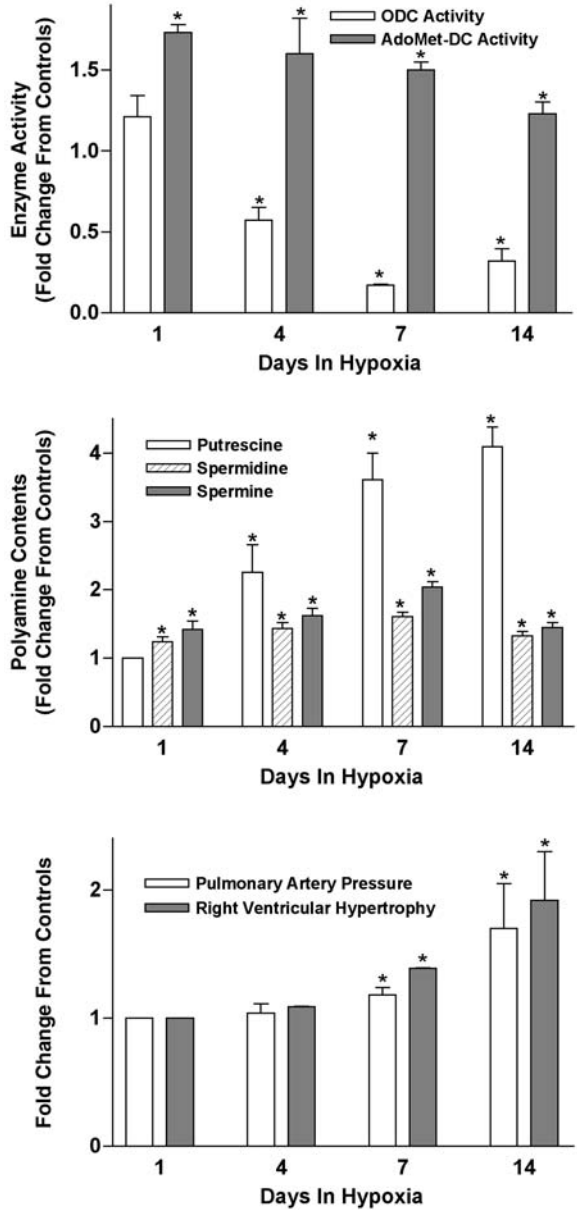

Fig. 3. Time-dependent changes in lung ODC, AdoMetDC, polyamine contents, mean pulmonary artery pressure, and right ventricular hypertrophy after continuous exposure to hypoxia (10% environmental oxygen). Control rats were exposed to room air (normoxia) and studied at each time point. Data reported are the fold change from control rats (mean ± the standard error). Control values were: ODC activity = 2.0 ± 0.12 nmoles/total lung weight/60 min; AdoMetDC

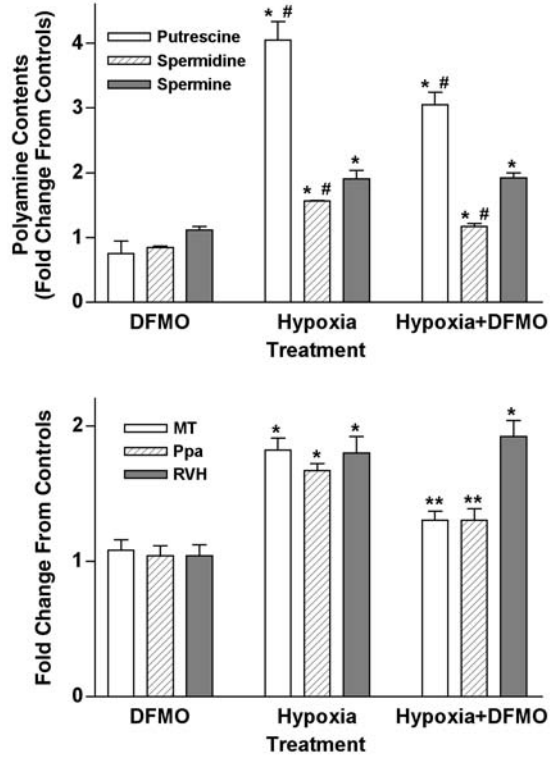

Fig. 4. Continuous DFMO treatment blunts chronic hypoxia-caused changes in rat lung polyamine contents and pulmonary arterial medial thickening (MT) and pressure (Ppa) and right ventricular hypertrophy (RVH). Rats were exposed to either normoxia or hypoxia for 21 d and 2% DFMO in the drinking water was given continuously starting 2 d before hypoxia. Control values were: putrescine = 20 ± 0.1 nmoles/left lung weight; spermidine = 270 ± 24 nmoles/left lung weight; spermine = 144 ± 6 nmoles/left lung weight; MT = 4.7 ± 0.36% of external diameter; Ppa = 15 ± 0.4 mm Hg; RVH = 0.25 ± .02 RV/LV+S. *Data differ significantly from normoxia group at $p < 0.05$; **data differ significantly from normoxia and hypoxia groups at $p < 0.05$; #data differ from DFMO at $p < 0.05$. $n = 6$ rats in each group. (Adapted from ref. *18*.)

putrescine and another for all three polyamines *(21)*. Intriguingly, hypoxia caused a selective, time-dependent induction of putrescine transport—neither spermidine nor spermine uptake were enhanced by hypoxia. Inhibition of hypoxia-stimulated putrescine uptake by a polyamine-specific transport inhibitor suppressed hypoxia-induced p38

activity = 3.7 ± 0.2 nmoles/total lung weight/60 min; putrescine = 16 ± 0.9 nmoles/left lung weight; spermidine = 223 ± 16 nmoles/left lung weight; spermine = 130 ± 9 nmoles/left lung weight; Ppa = 15.4 ± 1.0 mm Hg; RVH = 0.26 ± .001 RV/LV+S. Asterisk (*) indicates data differ from controls at $p < 0.05$. $n = 5$–6 rats in each group. (Adapted from refs. *17* and *19*.)

mitogen-activated protein kinase activation. These important observations suggest that a specific increase in the putrescine uptake pathway is necessary for hypoxia-induced activation of p38 mitogen-activated protein kinase—a growth regulatory protein kinase involved in cell adaptation to hypoxia. Further studies seem warranted to determine whether treatment with a polyamine uptake blocker can prevent development of hypoxia-induced pulmonary hypertension in rats.

Hypoxia not only regulates polyamine transport in both rat lung and cultured lung vascular cells, but also inhibits cultured PASMC *(22,23)* and PAEC *(24)* ODC activity, suggesting hypoxia decreases ODC activity in these cell types in the rat lung. Hypoxia time dependently inhibited ODC activity and mRNA content in cultured rat PAECs without altering antizyme contents. This finding is not particularly surprising because hypoxia inhibits ODC and augments polyamine transport in PAECs, whereas antizyme can inhibit both ODC and transport. The hypoxia-induced decrease in ODC activity was prevented by two strategies known to suppress proteasome-mediated ODC degradation: treatment with the proteasome inhibitor lactacystin or use of PAECs expressing a truncated ODC protein incapable of interacting with the proteasome. Both strategies prevented hypoxia-stimulated polyamine transport. These data suggest that in cultured rat PAECs, hypoxia-decreased ODC activity may be a stimulus initiating enhanced polyamine transport. Whether this apparently unusual regulatory process occurs in the hypoxic lung will require additional studies.

The mechanisms by which chronic hypoxia inhibits ODC activity and at the same time elevates polyamine contents in lung and cultured vascular cells appear to be unusual for the lung. In the other published studies on the regulation of lung polyamine content, enhanced ODC activity was a key determinant of polyamine content. For example, increased ODC activity accompanies increased polyamine content in postnatal lung development *(25)*, repair of hyperoxia-injured lungs *(26)*, and MCT-induced pulmonary hypertension. Furthermore, DFMO forestalls postnatal lung development *(25)*, inhibits repair of hyperoxic lung injury *(26)*, and prevents development of hypertensive pulmonary vascular disease in MCT-treated rats. Angiogenesis associated with growth of postnatal lungs *(25)* and repair of hyperoxic lungs *(26)* was significantly reduced by DFMO, further implicating polyamines as being required for pulmonary endothelial cell proliferation. Hypoxia may be a stimulus that has unique and complex polyamine regulatory properties.

5. Arginases and Pulmonary Hypertension

Arginase activity also regulates vascular cell polyamine contents. Arginases hydrolyze arginine to ornithine and thereby can regulate putrescine biosynthesis *(27)*. Alternatively, arginases compete with nitric oxide (NO) synthase for L-arginine to modulate the production of NO *(27–30)*, and by directly inhibiting ODC and AdoMetDC activity, NO can reduce polyamine biosynthesis *(31,32)*. In the case when arginase activity is enhanced, vascular smooth muscle *(33,34)* and endothelial cell *(35,36)* putrescine contents and proliferation rates are increased. When pulmonary artery endothelial cell arginase activity is inhibited, NO production is enhanced *(37)* and, in turn, NO inhibition of polyamine biosynthesis can prevent vascular cell proliferation

(31–33). In this context, the decreased NO production by injured or dysfunctional endothelial cells is believed to contribute to the inappropriate proliferation of both vascular and nonresident circulating cells in some vascular diseases, such as pulmonary hypertension.

The excessive lung vasoconstriction and vascular cell proliferation in pulmonary hypertensive humans is believed to be due partly to low NO production *(2–4,38,39)* and may occur because of elevated lung arginase activity *(40)*. Although the expression of lung NO synthase enzymes in patients with pulmonary hypertension was normal, these patients had low quantities of arginine and high serum arginase activity *(40)*. Immunohistochemistry revealed high arginase contents localized to the endothelium of hypertensive pulmonary arteries and arterioles. Compared with controls, PAECs isolated from human hypertensive lungs had higher arginase II protein expression and activity, and produced lower amounts of NO. It seems reasonable to postulate the combination of high arginase activity plus low NO may lead to excessive production of polyamines that subsequently promote vascular cell proliferation. Lung polyamine contents and their regulatory enzymes need to be evaluated in pulmonary hypertensive patients.

A few studies have evaluated L-arginine as potential therapy for pulmonary hypertensive patients because their NO levels are decreased. Overall, arginine therapy has had limited and varied success when administered acutely or for 1 wk, although long-term arginine therapy has not been studied in humans with pulmonary hypertension *(39)*. Another study reported that 10 patients with pulmonary hypertension secondary to sickle cell disease had decreased serum L-arginine contents and tended to have increased serum arginase activity, and 5 d of oral arginine therapy reduced pulmonary artery pressure *(41)*. In rat models of pulmonary hypertension, chronic oral L-arginine reduced pulmonary vascular remodeling and hypertension in hypoxia-exposed *(42)* and MCT-treated rats *(42,43)*, whereas D-arginine was not effective *(42)*. Although not examined in the above studies, L-arginine-enhanced NO production may have inhibited both ODC and AdoMetDC activity, and thereby reduced the pulmonary hypertension caused by hypoxia and MCT.

Arginases and polyamines may have important roles in the development of other lung diseases, such as fibrosis and asthma. Although nonvascular cell types are the primary targets in these diseases, remodeling of the lung vasculature occurs during the pathogenic process. In a rat model of pulmonary fibrosis, lung arginases I and II and ODC were increased and nitrogen oxides were decreased during the injury and repair response to hyperoxia *(44)*. Furthermore, immunostaining for arginase I and II was increased in perivascular regions. As previously described, DFMO inhibits the angiogenesis-associated repair of hyperoxic lungs *(26)*. The bleomycin mouse model of lung fibrosis has sustained increases in lung arginase and ODC mRNA contents *(45)*. In studies on asthma, arginase is increased in asthmatic lungs from humans *(46–48)* and experimentally induced allergic asthma in guinea pigs *(49)* and mice *(46)*. Of particular interest, putrescine was increased about twofold in allergen-challenged mouse lungs, although ODC mRNA was not changed from controls *(46)*. Lungs of human asthmatics have not been examined for changes in polyamines or ODC,

although asthmatics were reported to have elevated serum polyamine contents *(50)*. Although asthma is a disease primarily of the airways, neovascularization of the airways occurs. In this regard, large increases of arginase I mRNA were found in perivascular and peribronchial regions in the lungs of asthmatic mice *(46)*. Polyamines in the pulmonary circulation and airways may be important in asthma pathobiology and therapy and need further study.

6. Conclusions

It is evident that polyamines have important roles in the two most commonly studied animal models of pulmonary hypertension, although the mechanisms regulating the increased lung polyamine contents are clearly different. Increased lung ODC activity appears to be essential for the early pathogenic stages in the rat MCT model and to be important for the later stages of vascular remodeling. During the early stages, ODC activity regulates the responses of endothelial cells to injury. These responses likely include endothelial cell apoptosis, proliferation, barrier properties, and secretion of factors that create an inflammatory and thrombotic environment. In the later stages, ODC activity seems to contribute to the thickening of the medial and adventitial layers of the remodeling vasculature. In contrast to the MCT model, the role of ODC activity in the chronic hypoxia model is unclear because ODC activity is inhibited in response to hypoxia and, DFMO treatment reduces, but does not block, the pathological responses. Enhanced polyamine uptake and decreased efflux appear to be the predominant mechanisms, whereby hypoxia increases lung polyamine contents. Pulmonary arterial smooth muscle and endothelial cells seem to be primary targets of polyamine regulation in hypertensive pulmonary vascular disease. Based on these animal studies, polyamines regulate many pathogenic stages of pulmonary hypertension, and multiple mechanisms, including synthesis, degradation, and transport, control the contents of polyamines. The mechanisms regulating polyamines provide targets for multiple potential therapeutic strategies for pulmonary hypertension. Unfortunately, data supporting the importance of polyamines in pulmonary hypertension in humans are lacking, although increased arginase activity in pulmonary artery endothelial cells indicates polyamine contents may be elevated. Clinical studies are needed to evaluate lung polyamine contents and their regulation in patients with pulmonary hypertension. This new information will be critical for identifying whether polyamines are potential targets for the therapy of pulmonary hypertension.

References

1. Hoet, P. H. and Nemery, B. (2000) Polyamines in the lung: polyamine uptake and polyamine-linked pathological or toxicological conditions. *Am. J. Physiol. Lung Cell Mol. Physiol.* **278,** L417–L433.
2. Humbert, M., Morrell, N. W., Archer, S. L., et al. (2004) Cellular and molecular pathobiology of pulmonary arterial hypertension. *J. Am. Coll. Cardiol.* **43,** 13S–24S.
3. Budhiraja, R., Tuder, R. M., and Hassoun, P. M. (2004) Endothelial dysfunction in pulmonary hypertension. *Circulation* **109,** 159–165.
4. Newman, J. H., Fanburg, B. L., Archer, S. L., et al. (2004) Pulmonary arterial hypertension: Future directions: Report of a National Heart, Lung and Blood Institute/Office of Rare Diseases workshop. *Circulation* **109,** 2947–2952.

5. Olson, J. W., Altiere, R. J., and Gillespie, M. N. (1984) Prolonged activation of rat lung ornithine decarboxylase in monocrotaline-induced pulmonary hypertension. *Biochem. Pharmacol.* **33,** 3633–3637.
6. Olson, J. W., Hacker, A. D., Altiere, R. J., and Gillespie, M. N. (1984) Polyamines and the development of monocrotaline-induced pulmonary hypertension. *Am. J. Physiol.* **247,** H682–H685.
7. Orlinska, U., Olson, J. W., Gebb, S. A., and Gillespie, M. N. (1989) Acetylated polyamines in lungs from rats with monocrotaline-induced pneumotoxicity. *Fund. Appl. Toxicol.* **13,** 277–284.
8. Orlinska, U., Olson, J. W., and Gillespie, M. N. (1988) Polyamine content in pulmonary arteries from rats with monocrotaline-induced pulmonary hypertension. *Res. Commun. Chem. Pathol. Pharmacol.* **62,** 187–194.
9. Olson, J. W., Atkinson, J. E., Hacker, A. D., Altiere, R. J., and Gillespie, M. N. (1985) Suppression of polyamine biosynthesis prevents monocrotaline-induced pulmonary edema and arterial medial thickening. *Toxicol. Appl. Pharmacol.* **81,** 91–99.
10. Hacker, A. D. (1992) Inhibition of deoxyribonucleic acid synthesis by difluoromethylornithine. Role of polyamine metabolism in monocrotaline-induced pulmonary hypertension. *Biochem. Pharmacol.* **44,** 965–971.
11. Gillespie, M. N., Dyer, K. K., Olson, J. W., O'Connor, W. N., and Altiere, R. J. (1985) alpha-Difluoromethylornithine, an inhibitor of polyamine synthesis, attenuates monocrotaline-induced pulmonary vascular hyperresponsiveness in isolated perfused rat lungs. *Res. Commun. Chem. Pathol. Pharmacol.* **50,** 365–378.
12. Lipke, D. W., Arcot, S. S., Gillespie, M. N., and Olson, J. W. (1993) Temporal alterations in specific basement membrane components in lungs from monocrotaline-treated rats. *Am. J. Resp. Cell Mol. Biol.* **9,** 418–428.
13. Ruchko, M., Gillespie, M. N., and Olson, J. W. (2001) Suppression of ornithine decarboxylase activity prevents monocrotaline-induced rat lung fibronectin IIIA splicing. *FASEB J.* **15,** A858.
14. Ruchko, M., Gillespie, M. N., and Olson, J. W. (2001) Evidence that monocrotaline-induced apoptosis in rat lungs is dependent upon elevated-ornithine decarboxylase activity. *FASEB J.* **15,** A858.
15. Olson, J. W., Orlinska, U., and Gillespie, M. N. (1989) Polyamine synthesis blockade in monocrotaline-induced pneumotoxicity. *Biochem. Pharmacol.* **38,** 2903–2910.
16. Stenmark, K. R., Davie, N. J., Reeves, J. T., and Frid, M. G. (2005) Hypoxia, leukocytes, and the pulmonary circulation. *J. Appl. Physiol.* **98,** 715–721.
17. Olson, J. W., Hacker, A. D., Atkinson, J. E., Altiere, R. J., and Gillespie, M. N. (1986) Polyamine content in rat lung during development of hypoxia-induced pulmonary hypertension. *Biochem. Pharmacol.* **35,** 714–716.
18. Atkinson, J. E., Olson, J. W., Altiere, R. J., and Gillespie, M. N. (1987) Evidence that hypoxic pulmonary vascular remodeling in rats is polyamine dependent. *J. Appl. Physiol.* **62,** 1562–1568.
19. Shiao, R. T., Kostenbauder, H. B., Olson, J. W., Gillespie, M. N. (1990) Mechanisms of lung polyamine accumulation in chronic hypoxic pulmonary hypertension. *Am. J. Physiol.* **259,** L351–L358.
20. Babal, P., Manuel, S. M., Olson, J. W., and Gillespie, M. N. (2000) Cellular disposition of transported polyamines in hypoxic rat lung and pulmonary arteries. *Am. J. Physiol. Lung Cell Mol. Physiol.* **278,** L610–L617.
21. Ruchko, M., Gillespie, M. N., Weeks, R. S., Olson, J. W., and Babal, P. (2003) Putrescine transport in hypoxic rat main PASMCs is required for p38 MAP kinase activation. *Am. J. Physiol. Lung Cell Mol. Physiol.* **284,** L179–L186.

22. Haven, C. A., Olson, J. W., Arcot, S. S., and Gillespie, M. N. (1992) Polyamine transport and ornithine decarboxylase activity in hypoxic pulmonary artery smooth muscle cells. *Am. J. Respir Cell Mol. Biol.* **7,** 286–292.
23. Harrod, K. S., Olson, J. W., and Gillespie, M. N. (1996) Regulation of ornithine decarboxylase by hypoxia in pulmonary artery smooth muscle cells. *Am. J. Physiol.* **271,** L31–L37.
24. Babal, P., Ruchko, M., Ault-Ziel, K., Cronenberg, L., Olson, J. W., and Gillespie, M. (2002) N. Regulation of ornithine decarboxylase and polyamine import by hypoxia in pulmonary artery endothelial cells. *Am. J. Physiol. Lung Cell Mol. Physiol.* **282,** L840–L846.
25. Thet, L. A. and Parra, S. C. (1986) Role of ornithine decarboxylase and polyamines in early postnatal lung growth. *J. Appl. Physiol.* **61,** 1661–1666.
26. Thet, L. A., Parra, S. C., and Shelburne, J. D. (1984) Repair of oxygen-induced lung injury in adult rats. The role of ornithine decarboxylase and polyamines. *Am. Rev Resp. Dis.* **129,** 174–181.
27. Morris, S. M., Jr. (2004) Recent advances in arginine metabolism. *Curr. Opin. Clin. Nutr. Metab. Care* **7,** 45–51.
28. Kepka-Lenhart, D., Mistry, S. K., Wu, G., Morris, S. M., Jr. (2000) Arginase I: A limiting factor for nitric oxide and polyamine synthesis by activated macrophages? *Am. J. Physiol. Reg. Integr. Comp. Physiol.* **279,** R2237–R2242.
29. Meurs, H., Maarsingh, H.,and Zaagsma, J. (2003) Arginase and asthma: Novel insights into nitric oxide homeostasis and airway hyperresponsiveness. *Trends Pharmacol. Sci.* **24,** 450–455.
30. Mori, M. and Gotoh, T. (2004) Arginine metabolic enzymes, nitric oxide and infection. *J. Nutr.* **134,** 2820S–2825S; discussion 2853S.
31. Bauer, P. M., Buga, G. M., Fukuto, J. M., Pegg, A. E., and Ignarro, L. J. (2001) Nitric oxide inhibits ornithine decarboxylase via S-nitrosylation of cysteine 360 in the active site of the enzyme. *J. Biol. Chem.* **276,** 34,458–34,464.
32. Hillary, R. A. and Pegg, A. E. (2003) Decarboxylases involved in polyamine biosynthesis and their inactivation by nitric oxide. *Biochim. Biophys. Acta* **1647,** 161–166.
33. Ignarro, L. J., Buga, G. M., Wei, L. H., Bauer, P. M., Wu, G., and del Soldato, P. (2001) Role of the arginine-nitric oxide pathway in the regulation of vascular smooth muscle cell proliferation. *Proc. Natl. Acad. Sci. USA* **98,** 4202–4208.
34. Wei, L. H., Wu, G., Morris, S. M., Jr., and Ignarro, L. J. (2001) Elevated arginase I expression in rat aortic smooth muscle cells increases cell proliferation. *Proc. Natl. Acad. Sci. USA* **98,** 9260–9264.
35. Li, H., Meininger, C. J., Hawker, J. R., Jr., et al. (2001) Regulatory role of arginase I and II in nitric oxide, polyamine, and proline syntheses in endothelial cells. *Am. J. Physiol. Endocrinol. Metab.* **280,** E75–E82.
36. Li, H., Meininger, C. J., Kelly, K. A., Hawker, J. R., Jr., Morris, S. M., Jr., and Wu, G. (2002) Activities of arginase I and II are limiting for endothelial cell proliferation. *Am. J. Physiol. Reg. Integr. Comp. Physiol.* **282,** R64–R69.
37. Chicoine, L. G., Paffett, M. L., Young, T. L., and Nelin, L. D. (2004) Arginase inhibition increases nitric oxide production in bovine pulmonary arterial endothelial cells. *Am. J. Physiol. Lung Cell Mol. Physiol.* **287,** L60–L68.
38. Yuan, J. X. and Rubin, L. J. (2005) Pathogenesis of pulmonary arterial hypertension: The need for multiple hits. *Circulation* **111,** 534–538.
39. Badesch, D. B., Abman, S. H., Ahearn, G. S., et al. (2004) Medical therapy for pulmonary arterial hypertension: ACCP evidence-based clinical practice guidelines. *Chest* **126,** 35S–62S.

40. Xu, W., Kaneko, F. T., Zheng, S., et al. (2004) Increased arginase II and decreased NO synthesis in endothelial cells of patients with pulmonary arterial hypertension. *FASEB J.* **18,** 1746–1748.
41. Morris, C. R., Morris, S. M., Jr., Hagar, W., et al. (2003) Arginine therapy: A new treatment for pulmonary hypertension in sickle cell disease? *Am. J. Resp. Crit. Care Med.* **168,** 63–69.
42. Mitani, Y., Maruyama, K., and Sakurai, M. (1997) Prolonged administration of L-arginine ameliorates chronic pulmonary hypertension and pulmonary vascular remodeling in rats. *Circulation* **96,** 689–697.
43. Sasaki, S., Asano, M., Ukai, T., et al. (2004) Nitric oxide formation and plasma L-arginine levels in pulmonary hypertensive rats. *Resp. Med.* **98,** 205–212.
44. Que, L. G., Kantrow, S. P., Jenkinson, C. P., Piantadosi, C. A., and Huang, Y. C. (1998) Induction of arginase isoforms in the lung during hyperoxia. *Am. J. Physiol.* **275,** L96–L102.
45. Endo, M., Oyadomari, S., Terasaki, Y., et al. (2003) Induction of arginase I and II in bleomycin-induced fibrosis of mouse lung. *Am. J. Physiol. Lung Cell Mol. Physiol.* **285,** L313–L321.
46. Zimmermann, N., King, N. E., Laporte, J., et al. (2003) Dissection of experimental asthma with DNA microarray analysis identifies arginase in asthma pathogenesis. *J. Clin. Invest.* **111,** 1863–1874.
47. Morris, C. R., Poljakovic, M., Lavrisha, L., Machado, L., Kuypers, F. A., and Morris, S. M, Jr. (2004) Decreased arginine bioavailability and increased serum arginase activity in asthma. *Am. J. Resp. Crit. Care Med.* **170,** 148–153.
48. Fajardo, I., Svensson, L., Bucht, A., and Pejler, G. (2004) Increased levels of hypoxia-sensitive proteins in allergic airway inflammation. *Am. J. Resp. Crit. Care Med.* **170,** 477–484.
49. Meurs, H., McKay, S., Maarsingh, H., et al. (2002) Increased arginase activity underlies allergen-induced deficiency of cNOS-derived nitric oxide and airway hyperresponsiveness. *Br. J. Pharmacol.* **136,** 391–398.
50. Kurosawa, M., Shimizu, Y., Tsukagoshi, H., and Ueki, M. (1992) Elevated levels of peripheral-blood, naturally occurring aliphatic polyamines in bronchial asthmatic patients with active symptoms. *Allergy* **47,** 638–643.

II

Polyamines in Cellular Signaling of Apoptosis, Carcinogenesis, and Cancer Theraphy

12

Recent Advances in the Understanding of Mammalian Polyamine Catabolism

The Regulation and Potential Role of Polyamine Catabolism in Drug Response and Disease Processes

Robert A. Casero, Jr., Alison V. Fraser, Tracy Murray-Stewart, Amy Hacker, Naveen Babbar, Jennifer Fleischer, and Yanlin Wang

1. Introduction

As more data emerge, the significance of polyamine catabolism in polyamine homeostasis, drug response, and disease etiology is expanding. Importantly, the regulation and function of the polyamine catabolic pathway has emerged as a rational target for drug intervention in both chemotherapeutic and chemopreventive strategies. Mammalian intracellular polyamine catabolism had long been thought to be a two-step process primarily regulated by a rate-limiting acetyltransferase, spermidine/spermine N^1-acetyltransferase (SSAT), followed by the activity of a constitutively expressed acetylpolyamine oxidase (PAO). However, as recent reports have clearly demonstrated, mammalian polyamine catabolism also includes the activity of a previously unrecognized spermine oxidase (SMO/PAOh1). The production of reactive oxygen species (ROS) and other toxic products by these various polyamine catabolic enzymes can result in both useful and potentially dangerous consequences. This chapter will examine some of the most recent findings related to polyamine catabolism and will address the cloning and characterization of mammalian polyamine oxidases, including the newly discovered SMO/PAOh1. Additionally, further characterization of the highly regulated SSAT, as facilitated by many recent advances with transgenic models, will be discussed with respect to the potential role that it and the oxidases play in determining response to various drugs and stimuli. Although the polyamine catabolic pathway is well described and being studied in multiple organisms, this work will focus primarily on results directly related to mammalian systems, with special emphasis given to the relationship between polyamine catabolism and human disease. Specifically, data indicating that the induction of polyamine catabolism by specific antitumor polyamine analogs plays a

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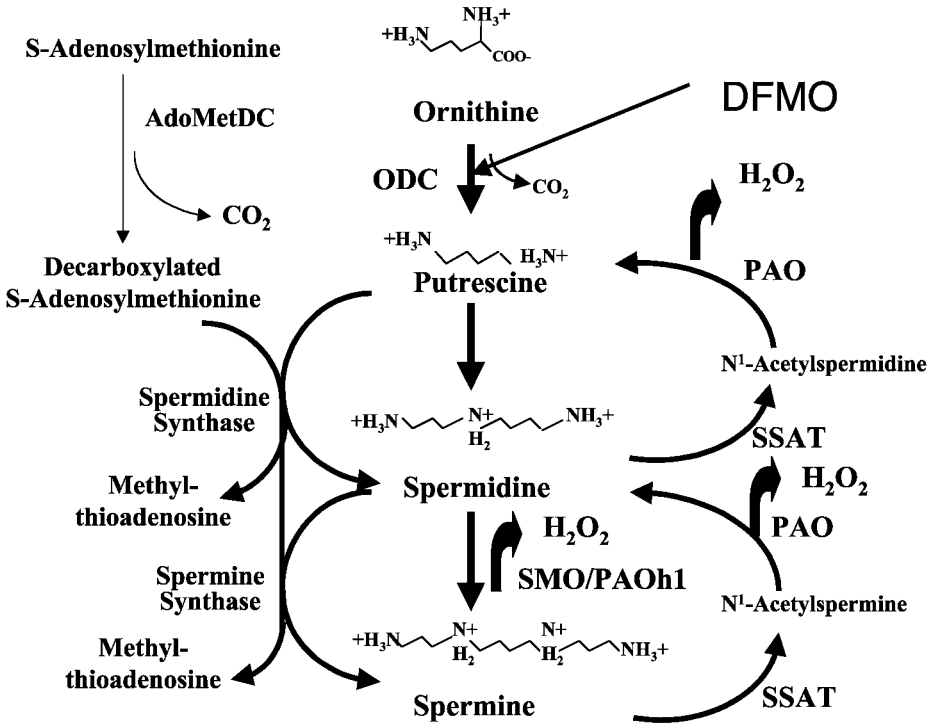


Fig. 1. The polyamine metabolic pathway. AdoMetDC, *S*-adenosylmethionine decarboxylase; DFMO, 2-difluoromethylornithine; ODC, ornithine decarboxylase; PAO, N^1-acetylpolyamine oxidase; SMO/PAOh1, spermine oxidase; SSAT, spermidine/spermine N^1-acetyltransferase.

direct role in determining drug response will be discussed. Also to be examined is the recent recognition that the oxidation of polyamines contributes to disease processes, and the potential targeting of polyamine catabolism as a strategy for chemoprevention.

2. Overview of Polyamine Metabolism

The mammalian polyamines—putrescine, spermidine, and spermine—are naturally occurring polycationic alkylamines. The polyamine metabolic pathway has been considered a target for antiproliferative drug development since it was discovered that polyamines are absolutely essential for cell proliferation. Effective inhibitors now exist for virtually all of the biosynthetic enzymes of the polyamine pathway (Fig. 1). Much of the work in the last 30 yr or more has focused on inhibiting the biosynthetic enzymes that control polyamine production, particularly the rate-limiting enzymes of biosynthesis, ornithine decarboxylase (ODC) and *S*-adenosylmethionine decarboxylase (AdoMetDC). The most widely studied inhibitor of polyamine biosynthesis is 2-difluromethylornithine (DFMO), an enzyme-activated inhibitor of ODC *(1,2)*. Though DFMO was not effective as a single agent for cancer, it has seen considerable success

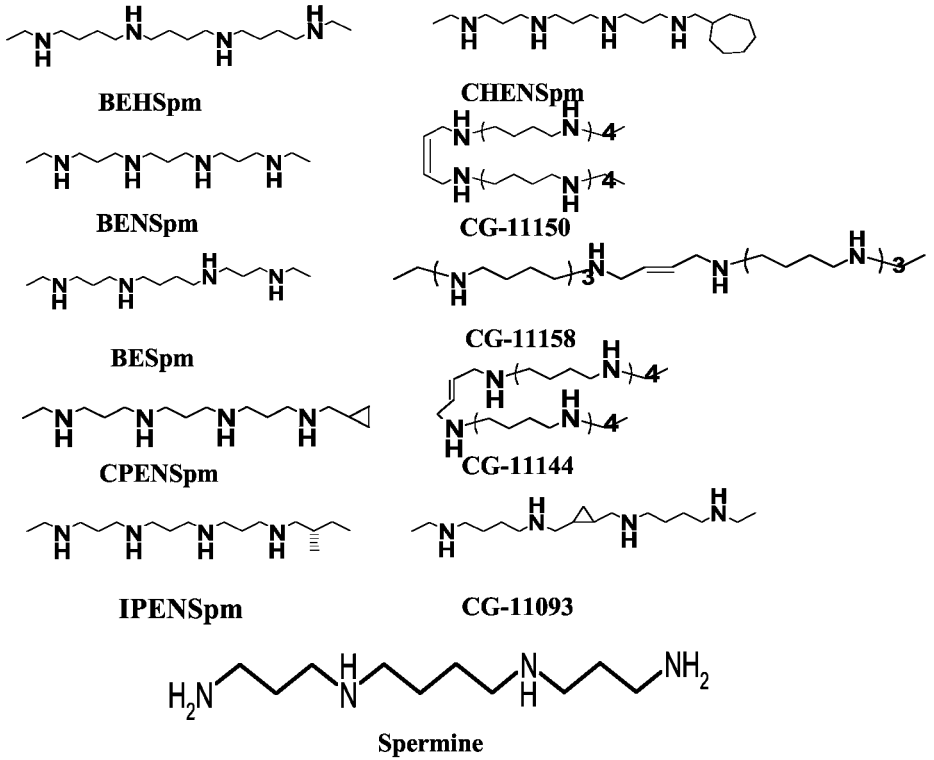


Fig. 2. Select polyamine analogs. BENSpm, N^1,N^{11}-*bis*(ethyl)norspermine; CPENSpm, N^1-ethyl-N^{11}-(cyclopropyl)methyl-4,8,diazaundecane; CHENSpm, N^1-ethyl-N^{11}-(cycloheptyl)methyl-4,8,diazaundecane; IPENSpm, (S)-N^1-(2-methyl-1-butyl)-N^{11}-ethyl-4,8,diazaundecane.

as an antitrypanosomal drug targeting the organism responsible for African sleeping sickness and is being clinically evaluated as a chemopreventive agent *(3)*.

Although the efficacy of polyamine biosynthetic inhibitors has been limited in cancer treatment, their study has provided a wealth of data demonstrating that targeting polyamine metabolism is a rational approach for antineoplastic therapy. Recently, an alternative approach to specifically inhibiting polyamine biosynthetic enzymes has emerged. The development of agents that mimic the autoregulatory function of the polyamines but are unable to functionally substitute in the growth-promoting roles has proceeded. Several classes of polyamine analogs have been synthesized and evaluated in multiple in vitro and in vivo model systems, and in some cases, clinical trials *(4–12)*. Most polyamine analogs have the ability to downregulate the biosynthetic enzymes ODC and AdoMetDC, compete with the natural polyamines for uptake into cells, and many also upregulate polyamine catabolism.

In early studies of the polyamine analogs (Fig. 2), including N^1, N^{11}-*bis*(ethyl)norspermine (BENSpm) that was synthesized by Bergeron and colleagues, we observed that

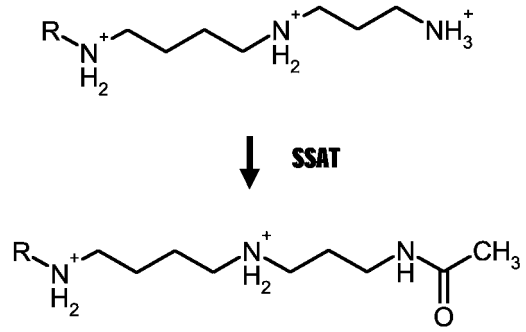


Fig. 3. SSAT activity. SSAT transfers the acetyl moiety from acetyl coenzyme A to the N^1-position of either spermidine (R = H) or spermine (R = $C_3H_9N_1$).

activity of the catabolic enzyme, SSAT, could be induced several thousand-fold in a cell and tumor type-specific manner *(13–15)*. Because the high induction of SSAT appeared to be linked to the antiproliferative effects of multiple analogs, the study of polyamine catabolism as a target for therapy intensified *(16–18)*. These initial findings not only produced an increased interest in SSAT specifically, but also in polyamine catabolism in general. Since the time of the original discovery linking induced polyamine catabolism to drug sensitivity, the completion of numerous biochemical and molecular studies have facilitated a better understanding of the broader significance of normal and aberrant polyamine catabolic processes. It should be noted that there are other important enzymes involved in the catabolism of the polyamines and related compounds, including di- and monoamine oxidases, and copper-containing serum amine oxidases. Although these enzymes clearly can be important in many facets of polyamine catabolism, they will not be covered here as they have been reviewed by Seiler *(17)*.

3. Spermidine/Spermine N^1-Acetyltransferase

3.1. An Overview of SSAT Enzyme Function and the Regulation of Its Expression

The polyamine biosynthetic pathway is essentially irreversible; however, it was determined relatively early that spermidine and spermine could be converted back to putrescine. The rate-limiting step of one back conversion pathway is SSAT *(19)* (Fig. 3). SSAT catalyzes the transfer of the acetyl moiety from acetyl coenzyme A (acetyl CoA) to the N^1 position of either spermidine or spermine creating an acetylated polyamine that can be excreted from the cell or serve as a substrate for the acetylpolyamine oxidase, PAO (*see* Subheading 4.). The K_m for acetyl CoA of the human enzyme is in the low m*M* range and the K_m for spermine and spermidine are 5–60 m*M* and 55–140 m*M*, respectively. Although SSAT protein is generally not present in cells in detectable amounts, it is readily detected after induction by various stimuli, including toxic agents, growth factors, polyamines, and, as will be discussed in greater detail, polyamine analogs *(19)*.

Our cloning of the human *SSAT* gene made it the first of the mammalian polyamine catabolic enzymes to be cloned, thus becoming the best characterized of the catabolic

enzymes *(20)*. *SSAT* from multiple species have been subsequently cloned and all demonstrate high homology to the human gene *(21,22)*. Human *SSAT* is located at chromosome Xp22.1 and consists of six exons coding for a 20-kD protein of 171 amino acids that is likely active as a homodimer *(20,23–25)*. It should be noted that alternative splice variants have been implicated for the *SSAT* gene; however, the significance of these variants has yet to be determined *(26,27)*.

Initial studies demonstrating that SSAT could be induced in a phenotype-specific manner by certain polyamine analogs suggested that this rapid induction was primarily a result of analog-induced transcription *(20)*. However, it soon became evident that SSAT expression is regulated at virtually every step from transcription to posttranslational stabilization of the protein.

In responsive cell types, typified most dramatically by several human non-small-cell lung cancer and specific human melanoma cell lines, transcription of SSAT increases only modestly (three- to sevenfold), whereas protein and activity can increase more than 2000-fold, directly implicating significant posttranscriptional regulation in response to specific analogs *(28,29)*. Porter and colleagues demonstrated that in human melanoma cells, treatment with polyamine analogs leads to a stabilization of SSAT messenger RNA (mRNA), as well as SSAT protein, as determined by increased half-lives of both *(28,30)*. The stabilization of SSAT mRNA appears to result, in part, from increased translational efficiency as indicated by an increase in polysome-associated SSAT mRNA *(31)*. Coleman et al. defined the mechanism by which the natural polyamines and the polyamine analogs stabilize SSAT protein that leads to the huge increases in the enzyme levels observed in analog-induced cells. Site-directed mutagenesis identified the carboxyl terminal domain consisting of an amino acid sequence, MATEE, as being critical for both activity and stabilization of the SSAT protein *(32,33)*. Although SSAT protein has been demonstrated to have a very short half-life (<30 min), unlike most other rapidly degraded proteins, SSAT does not possess a discernible PEST *(34)*. Current data suggest that the MATEE sequence may be able to function in the role of the classical PEST sequence *(33)*. Most importantly, it has been demonstrated that polyamine analogs can prevent the efficient ubiquitination of SSAT, thus stabilizing the protein from proteosomal degradation *(35)*.

In addition to the SSAT protein domains important for polyamine and polyamine analog binding and stabilization, the amino acid residues critical for acetyl CoA binding have been identified *(25,36)*.

3.2. Transcriptional Regulation of SSAT

Although there is significant posttranscriptional regulation of SSAT in response to the antitumor polyamine analogs, the increase in SSAT expression is first dependent on elevated mRNA levels downstream of increased transcription *(28,29)*. This increase in *SSAT* transcription in response to polyamines and polyamine analog exposure is generally only three- to sevenfold. However, *SSAT* transcription may be the primary point of control in response to natural physiological changes in polyamine pools because the natural polyamines are substrates for SSAT, and thus, are limited in their ability to posttranscriptionally regulate the enzyme.

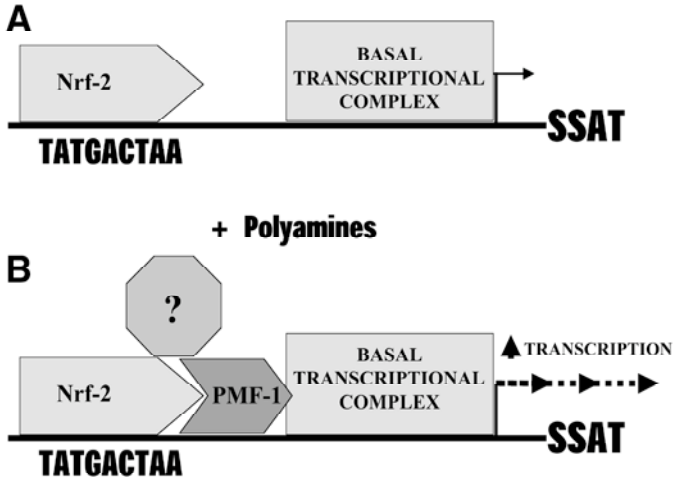


Fig. 4. Proposed model for polyamine and polyamine analog activation of *SSAT* transcription. In the absence of excess polyamines or analogs, Nrf-2 bound to the PRE, but basal transcription remains low. In the presence of inducers, PMF-1 expression increases, binds to Nrf-2, and drives transcriptions of *SSAT*. However, the possibility exists that yet to be identified transcription cofactors (as indicated by **?**) may modify *SSAT* transcription in response to polyamines and their analogs.

The inducible transcription of human SSAT was found to be under the control of a nine base pair *cis*-element 5¢-TATGACTAA-3¢, in a 31-base context starting –1492 base pairs upstream from the transcriptional start site *(37)*. This element, referred to as the polyamine responsive element (PRE), was found to be constitutively occupied only in analog-responsive cells by the DNA-binding transcription factor, NF-E2-related transcription factor-2 (Nrf-2). There was no evidence to indicate that treatment with analogs or the natural polyamines altered the binding of Nrf-2 to the PRE. These results suggested the possibility that additional factors were necessary for analog responsiveness.

Therefore, using a yeast two-hybrid strategy an analog-inducible transcription cofactor, named polyamine-modulated factor 1 (PMF-1) was identified *(38)*. This 20-kDa protein is unable to bind DNA directly because it lacks a functional DNA-binding domain. However, it appears to modulate the transcriptional activation of SSAT via binding to Nrf-2 through a unique leucine zipper/coiled-coil interaction. Interestingly, PMF-1 expression is similar to that of SSAT in that its inducible expression appears to be cell type- and agent type-specific (Fig. 4).

It is also important to note that Nrf-2 and PMF-1 may not be the only factors involved in the transcriptional regulation of SSAT. Nrf-2 is known to have several other binding partners, including the small Maf family transcription cofactors and the Keap1 cytosolic regulatory protein *(39–42)*. Further, PMF-1 has been found to bind to the human homolog of the 7a subunit of the *Arabidopsis* Cop 9 signalosome *(43)*. Consequently, further study will be required to determine if there are other components contributing to transcriptional regulation of SSAT. Such studies are important because

the analog-induced expression of SSAT has been linked to the responsiveness of specific tumor cells and a better understanding as to how SSAT transcription is regulated has the potential of improving drug design. It is also likely that by gaining a better understanding as to how *SSAT* transcription is controlled, such investigation will allow *SSAT* to be established as a model gene through which the potential ways that polyamines and their analogs can affect specific gene expression can be investigated.

3.3. Role of SSAT Activity in Cellular Response to Polyamine Analogs

The observations that link SSAT activity and cellular response after exposure to specific polyamine analogs have stimulated interest not only in polyamine catabolism in general, but also into the understanding of how this enzyme's activity can affect cellular survival.

The first indication that highly induced SSAT activity was associated with cytotoxicity was demonstrated in human non-small-cell lung cancer cells *(13,14)*. This phenotype-specific response to specific antitumor polyamine analogs was subsequently observed in other human tumor cell types *(44–50)* and confirmed in several in vivo models, demonstrating a significant increase in SSAT activity in response to analog treatment of sensitive tumor types. Importantly, the increased activity observed in vivo was generally specific for the tumor cells *(51–57)*. Although transient increases in kidney and liver SSAT activities were observed, these enzyme levels rapidly returned to normal after treatment was discontinued. In comparison, the tumor-specific increase in SSAT was not only higher, but persisted considerably longer after cessation of treatment. The tumor selectivity can also be demonstrated for human tumors *in situ*. When human non-small-cell lung cancer explants from biopsies are exposed, in vitro, to 10 m*M* BENSpm for 24 h, there is a significant increase in tumor-specific induction of SSAT as determined by specific antibody staining (Fig. 5). These results clearly implicate the activity of SSAT as being associated with the observed tumor-selective cytotoxicity; however, it was not clear whether the cytotoxicity was the result of increased SSAT enzyme activity, decreased polyamine pools, direct activity by the analog, or some combination of each.

Another indication of a direct link between SSAT and drug response was observed in human lung tumor cells of female origin that possess two active copies of the X-linked *SSAT*. The NCI H727 carcinoid line expressing both copies were significantly more sensitive to the cytotoxic effects of the analog BESpm than female cells that only expressed a single copy *(58)*. Additional data indicating that increased SSAT expression alone could lead to growth inhibition came when human SSAT was overexpressed in *Escherichia coli* leading to a near complete depletion of spermidine and decreased bacterial growth *(59)*. However, the clearest evidence indicating a direct role for SSAT activity in the cellular response to the polyamine analogs was observed by Vujcic et al. using a Tet-repressible SSAT expression system in MCF-7 human breast cancer cells *(60)*. Derepression of the expression vector produced a 20-fold increase in SSAT mRNA and a 10-fold increase in enzyme activity. This increased activity was accompanied by a significant decrease in growth rate. When increased SSAT expression was combined with BENSpm treatment, the SSAT-overexpressing cells were significantly more sensitive to the analog than wild-type

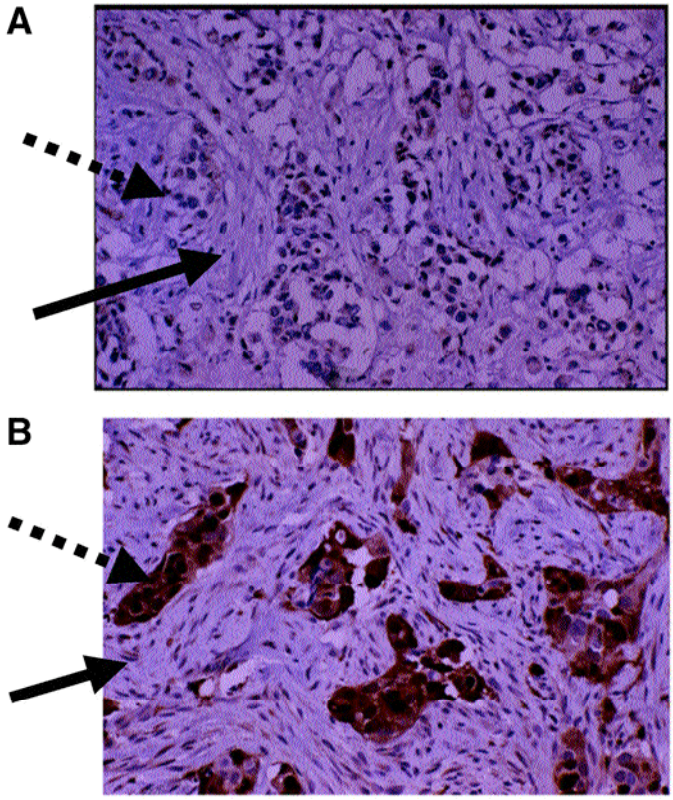


Fig. 5. Immunohistochemical staining of a human adenocarcinoma explant after exposure to BENSpm. Adenocarcinoma explants derived from the same aseptic core biopsy were divided into thin (approx 1 mm) sections and incubated in medium containing 10 m*M* BENSpm **(B)** or control medium **(A)** for 24 h. After incubation, tissues were fixed and imbedded in paraffin and processed with a specific anti-SSAT antibody as we have previously reported *(54,55)*. Note the intense staining of only the tumor cells (dashed arrow) in the analog-treated sample **(B)**. No staining was observed in the adjacent normal cells (solid arrow) in the treated sample and no staining was observed in either the tumor (dashed arrow) or normal (solid arrow) in the untreated sample **(A)**.

MCF-7 cells or cells containing the expression vector, but not derepressed. Similar results were observed in a normally BENSpm-resistant human small-cell lung cancer line transfected with a constitutively expressing SSAT construct *(61)*.However, extreme increases in enzyme protein and activity have been found to be limited to those cells, transfected or not, that are treated with analog, consistent with a major regulatory step of SSAT superinduction being at the level of protein stabilization.

It is important to note that growth inhibition associated with high SSAT protein expression is dependent on SSAT catalytic activity. McCloskey et al. *(62,63)* demonstrated that Chinese hamster ovary (CHO) cells expressing even high levels of an SSAT

protein containing a L156F mutation were resistant to the growth inhibitory effects of several polyamine analogs. Additionally, this mutation resulted in a protein that was not capable of being stabilized by the polyamine analogs.

In addition to growth inhibition, apoptosis associated with increased polyamine catabolism has been observed in several systems. However, the precise mechanisms responsible for the induced apoptosis remain unclear and may be cell type-specific. It is clear that in some cell systems the induction of apoptosis is associated with, and may be dependent on, the induction of SSAT. The best demonstration of this to date are studies reported by Chen et al. using transient transfection of the analog-sensitive human SK-MEL-28 melanoma cells with small interfering RNA targeting SSAT *(64)*. Their results clearly indicate that in this melanoma cell system, the induction of SSAT is necessary for the early downstream events of apoptosis to occur. Unfortunately, because these were transient transfection studies, the longer term effects of SSAT knockdown were not analyzed.

A gene with homology to SSAT has recently been reported and is designated as *SSAT2 (65,66)*. However, because it does not appear that the protein encoded by this gene plays a significant role in polyamine metabolism, it will not be discussed further here *(66)*.

3.4. SSAT Transgenic Models

Cellular transfection models have contributed significantly to our understanding of the importance of SSAT activity in the maintenance of polyamine homeostasis and its potential role in drug response. However, a wealth of information has been derived from a variety of recent transgenic systems *(67–81)*. One general feature emerging from these studies that is consistent with in vitro observations is the fact that extremely high SSAT activity is a result of posttranscriptional regulation by polyamine analogs. Without analog exposure, very large increases in SSAT mRNA can occur with only small increases in enzyme protein and activity *(67,69,81)*. Another interesting finding in the transgenic animals was that when ODC and SSAT were both overexpressed in the same animals, polyamine catabolism controlled by SSAT overrode polyamine biosynthesis in determining intracellular polyamine concentrations and polyamine metabolites *(80)*. These data are completely consistent with results observed in MCF-7 cells induced to overexpress SSAT, then supplemented with exogenous polyamines *(60)*. Specifically, the added polyamines could not overcome the increased SSAT activity to restore polyamine concentrations to normal levels. Taken together, these results suggest that polyamine catabolism is the major control point of polyamine homeostasis.

It is notable that high systemic overexpression of SSAT results in a mouse that has a similar outward appearance to mice overexpressing ODC *(82)*. In both cases, the mice lose their hair at an early age and have progressive and severe skin wrinkling with age. Because both genotypes result in high putrescine levels in the skin, and because DFMO treatment of the ODC transgenics prevents the skin phenotype, high putrescine levels have been implicated in the ensuing phenotype.

A more recent and targeted SSAT transgenic mouse has been created using the bovine K6 promoter to target SSAT expression to the epidermal keratinocytes in the hair follicle *(72)*. Unlike the previous models systemically expressing multiple copies of SSAT, these

animals did not lose their hair and, when left untreated, did not exhibit significant changes in SSAT activity or polyamine concentrations in the skin. However, when these mice were challenged with a two-stage tumorigenesis protocol, the transgenic animals were considerably more sensitive to the dimethylbenzanthracine/12-*O*-tetradodecanoylphorbol-13-acetate (DMBA/TPA) treatment than the wild-type mice, resulting in a much greater tumor (98 to 60% after 19 wk of promotion). More importantly, a 31% incidence of malignant carcinomas was observed in the K6-SSAT mice, whereas no malignant tumors were observed in the identically challenged wild-type mice.

The results in the K6-SSAT transgenic mice appear to be in contrast to those observed by Pietilä et al., in which systemic overexpression of SSAT reduced the number of tumors produced by a similar two-stage protocol. This apparent contradiction could be a result of the different mouse strains used by the investigators, or the result of the specific targeting of the epidermal follicles in the K6-SSAT system, rather than systemic overexpression. More experimentation will be necessary to resolve this apparent contradiction.

In addition to the depletion of polyamines and the production of reactive oxygen species, Kee et al. have recently demonstrated another potential mechanism by which activated SSAT can lead to growth inhibition or cell death in a cell type-specific manner *(76,83)*. Using both in vitro and in vivo prostate models, the high expression of an exogenous *SSAT* gene leads to a decrease in growth rate that is associated with a significant depletion of acetyl CoA pools. This correlation was demonstrated in both prostate cancer cells and in prostates from TRAMP/SSAT double transgenic mice. In the double transgenic model, the prostate tissue in general is reduced in size, and the tumor progression appears limited based on histopathological scores. The authors propose that the reduction of acetyl CoA pools is owing to an accelerated flux through the polyamine metabolic pathway, resulting from a compensatory increase in polyamine biosynthesis in response to increased SSAT activity. This hypothesis is consistent with their in vitro findings using the LNCaP prostatic cancer cell line in which acetyl CoA depletion by SSAT overexpression could be prevented if the flux through the polyamine metabolic pathway was blocked by inhibiting biosynthesis with DFMO *(83)*. Although it would be preferable to see the results of these studies confirmed using a prostate-targeted SSAT expression mode, these results may have great significance for the development of therapeutic agents targeting SSAT induction in the prostate.

3.5. Expression of SSAT in Response to Stimuli Other Than Polyamines or Polyamine Analogs

Clearly, the transcriptional and posttranscriptional regulation of SSAT by the cell in response to the polyamines and their analogs represent highly regulated processes that are becoming well documented. Although it has long been known that multiple conditions, including toxins, hormones, and various other cellular stresses can lead to increased SSAT expression *(19)*, several previously unidentified inducers have been found to elevate cellular SSAT levels.

Sulindac, a nonsteroidal anti-inflammatory drug is metabolized into two active derivatives, a sulfide that is an effective cyclo-oxygenase (COX) inhibitor and a sul-

fone. Both metabolites are effective in inhibiting colon cancer cell growth. Surprisingly, the sulfone derivative was found to increase the transcription of SSAT through a peroxisome proliferator-activated receptor (PPAR) response element bound by PPARg *(84)*. The results of this study suggest that apoptosis induced by sulindac sulfone is COX-independent, and that PPAR-dependent activation of SSAT leads to polyamine depletion through acetylation and export. Although further study will be necessary to determine the relative effectiveness of the COX-independent induction of apoptosis as compared with the COX-dependent effects, it does open an entirely new avenue for SSAT-associated growth inhibition.

A series of recent reports suggest that specific antitumor agents structurally unrelated to the polyamines are also capable of upregulating the expression of SSAT mRNA *(85–87)*. Using a gene array analysis strategy, Maxwell et al. identified SSAT as being among a select number of genes whose expression was significantly increased in MCF-7 breast cancer cells after exposure to 5-fluorouracil (5-FU). However, because increases in SSAT mRNA have frequently been observed without concurrent increases in activity, and because activity was not measured in this system, the functional significance of the increased mRNA in response to 5-FU alone is not known.

Results from a series of studies in the colon cancer cell line HCT116 suggest that 5-FU can synergize with BENSpm treatment in regard to SSAT expression *(87)*. When 5-FU was used in combination with BENSpm, there were synergistic increases in both SSAT mRNA and apoptosis, as well as decreases in spermidine and spermine concentrations. These results are consistent with observations by Hahm et al. in MCF-7 cells treated with the combination of 5-FU and SSAT-inducing polyamine analogs *(88)*. Unfortunately, none of these investigators reported SSAT activity after drug treatment.

In a recent study, Hector et al. examined the combination of oxaliplatin and BENSpm in multiple cell types, including melanoma and ovarian cancer cells. This combination produced a clearly synergistic increase in SSAT mRNA and enzyme activity, and resulted in significantly more growth inhibition than when either drug was used as a single agent *(86)*. Most importantly, this study suggests a likely mechanism for the observed synergy. Specifically, it appears that the cytotoxic agent leads to a substantial increase in SSAT mRNA, but without a subsequent elevation of SSAT activity unless the polyamine analog is present to both enhance translation and stabilize the protein. The result is that substantially lower concentrations of each compound can be used to produce much greater growth inhibitory effects, potentially increasing the therapeutic index of the combination.

Taken together, the results from studies demonstrating that compounds other than polyamines or their analogs are capable to significantly regulate SSAT expression suggest that a judicious combination of agents may be used to increase the therapeutic efficacy of both polyamine- and nonpolyamine-targeted agents. Combining compounds targeting polyamine metabolism with traditional chemotherapeutic agents may hold the greatest promise for use in many polyamine pathway-targeting therapies.

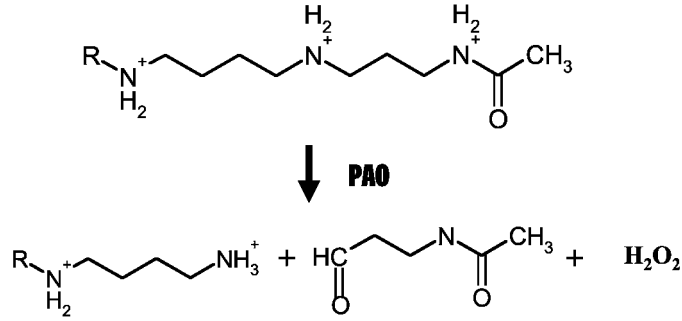


Fig. 6. PAO activity. The FAD-dependent PAO oxidizes either N^1-acetylspermidine (R = H) or N^1-acetylspermine (R = $C_3H_9N_1$), producing putrescine or spermidine, *N*-acetyl-3-aminopropanal, and H_2O_2.

3.6. SSAT Expression in Response to Stress

The *SSAT* gene has been described as a stress response gene and the protein was originally purified from rat liver stimulated with the toxic stressor, carbon tetrachloride *(19,89–91)*. Several more recent reports implicate SSAT as either a marker or mediator of ischemia/reperfusion-associated injury in multiple organ systems *(92–98)*. Although the mechanism of increased SSAT-associated injury has not been elucidated, in vitro results using HEK-293 kidney cells indicate that increased SSAT activity and the downstream oxidation of the acetylated polyamines by PAO lead to the production of toxic H_2O_2 *(98)*. This mechanism is similar to what has been suggested for some cellular responses to the polyamine analogs *(99,100)*. However, it should be emphasized that in this ischemia/reperfusion model, spermine oxidase activity is also highly induced and may play a significant role in the observed events.

4. N^1-Acetylpolyamine Oxidase

4.1. PAO Properties, Function, and Expression

The first description of purified rat liver PAO implicated it as a predominantly peroxisomal enzyme capable of oxidizing spermidine and spermine; however, it was much more active in the presence of benzaldehyde *(101)*. The recognition that the N^1-acetylpolyamines were the preferred substrates of PAO led to the belief that polyamine catabolism was a two-step process *(102–105)*, rate-limited by the acetylation of polyamines by SSAT *(19)*. PAO has been excellently reviewed by several authors *(17,106–112)*. However, only recently have mammalian PAOs been cloned and characterized *(113–115)*. Therefore, the focus here will be primarily on the most recent findings relevant to the better understanding of PAO provided by the cloning of the mammalian enzyme.

PAO catalyzes the oxidation of N^1-acetylspermine and N^1-acetylspermidine to spermidine and putrescine, respectively (Fig. 6). Both reactions occur by cycling the

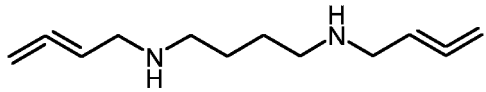
enzyme-bound FAD to the reduced $FADH_2$, and in the presence of molecular oxygen, producing H_2O_2 and 3-acetoamidopropanal *(114)*.

PAO possesses a terminal peroxisomal localization sequence (-PRL) consistent with the peroxisomal localization of PAO determined by Holtta *(101,114,116)*, although this targeting sequence does not preclude the existence of a cytosolic protein. It should be noted that both Wu et al. and Vujcic et al. have reported three mammalian PAOs, each of a different amino acid size, including a mouse PAO reported as 504 amino acids, a bovine protein of 452 amino acids, and the human protein of 511 amino acids. Additionally, 12 splice variants of the human PAO have been identified *(115)*. The existence of multiple splice variants may explain, in part, the various protein sizes initially attributed to PAO isozymes *(101,106,117)*. Another possibility is that some of the previous purifications may have resulted in a mixture of the different polyamine oxidases of similar size. The major human splice variant of spermine oxidase (SMO/PAOh1) codes for a 551 amino acid protein with both high-domain homology and amino acid identity (39%) with the 511 amino acid PAO. Thus some of the confusion with regard to substrate specificity in the earliest reports of PAO may best be explained as the result of a mixture of two similar-size proteins.

The purified human and mouse PAOs each have high catalytic activity (K_{cat} = 4.5–31.7 s^{-1}) and affinities for the N^1-acetylated polyamines (K_m = 0.85–1.78 m*M*), with the highest affinity being reported for N^1-acetylspermine. The substrate preference for PAO appears to be N^1-acetylspermine > N^1-acetylspermidine > N^1, N^{12}-diacetylspermine >>> spermine *(113–115)*.

4.2. Analogs as Substrates for PAO

Although the mammalian PAOs preferentially oxidize acetylated polyamines, it has long been assumed that they may also oxidize specific polyamine analogs *(118,119)*. Such activity could obviously affect the efficacy of those compounds when used against tumors expressing high PAO activity. However, the determination of which oxidases were active on what compounds awaited the identification and analysis of the individual enzymes. Wu et al. and Vujcic et al. both provide convincing evidence that the symmetrically substituted polyamine analogs typified by BENSpm are effective substrates for PAO *(113,114)*. Additionally, we have demonstrated that recombinant human PAO not only effectively oxidizes the symmetrically substituted analogs, but also efficiently uses the unsymmetrically substituted polyamines as substrates. Consistent with the results of Vujcic et al., the unsymmetrically substituted analogs used in our studies appear to be oxidized at the interior nitrogen, producing the appropriately substituted spermidine analog, rather than the predicted de-ethylation product *(113,115,118)*. However, in contrast to the observations of Vujcic et al., there was no evidence of PAO activity when BEHSpm was used as a substrate. In fact, none of the analogs possessing aminobutyl terminal moieties were oxidized by the recombinant enzyme *(115)*. The basis for this difference is not clear, although in our studies, purified, recombinant protein was used, whereas Vujcic et al. used lysates from cells transfected with PAO expression vectors *(113,115)*.



MDL 72,527

Fig. 7. The structure of the polyamine oxidase inhibitor, N^1, N^4-*bis*(2,3-butadienyl)-1,4-butanediamine (MDL 72,527).

It was recently demonstrated that when human A549 lung adenocarcinoma cells were transfected with PAO, those clones expressing high levels of PAO became wholly resistant to all of the analogs determined to be substrates of PAO *(115)*. These data are entirely consistent with the previous observations of Lawson et al. demonstrating the resistance of CHO cells to CHENSpm, based on high oxidase activity in the CHO cells *(119)*. These data, in combination with other reports *(120–122)* suggest that the levels of PAO activity within cells may have a profound effect on the sensitivity of various tumors to specific antitumor polyamine analogs *(16)*.

4.3. Inhibitors of PAO

Even before the mammalian enzyme had been cloned and fully characterized, attempts were made to specifically inhibit PAO to understand the significance of its activity. N^1, N^4-*b i s*(butadenyl)-1,4-diaminobutane (MDL 72,527; Fig. 7) was the first inhibitor synthesized specifically for this purpose *(123)*. It was demonstrated that the cotreatment of cells with MDL 72,527 and specific analogs could enhance the activity of those compounds, presumably because MDL 72,527 inhibits their metabolism *(124)*. However, MDL 72,527 has recently been demonstrated to be somewhat less specific in its inhibition than previously thought, as it has been found to be a highly effective inhibitor of the newest member of the polyamine catabolic pathway, SMO/PAOh1 *(125–128)*.

Other compounds that are effective inhibitors of human PAO include several antitumor polyamine analogs known as oligoamines, which were synthesized by Frydman and colleagues *(10,115,129–132)*. The mechanism by which these compounds inhibit PAO is unknown; however, it is significant that they also efficiently inhibit SMO/PAOh1 *(126)*.

Although the activity of mammalian PAO has been predicted and studied for several years, only now with its cloning and characterization and with the discovery of the closely related spermine oxidase, will it be possible to fully understand the role of PAO in polyamine homeostasis and drug response. The next few years should be very telling in determining the important roles this enzyme plays in both normal and neoplastic cells.

5. Spermine Oxidase (SMO/PAOh1)

5.1. SMO Regulation, Expression, and Potential Role in Drug Response

SMO/PAOh1 is the most recent of the polyamine catabolic enzymes to be discovered. This enzyme catalyzes the oxidation of spermine to spermidine, 3-aminopropanal, and H_2O_2 (Fig. 8). Our laboratory was the first to clone this oxidase (and named it

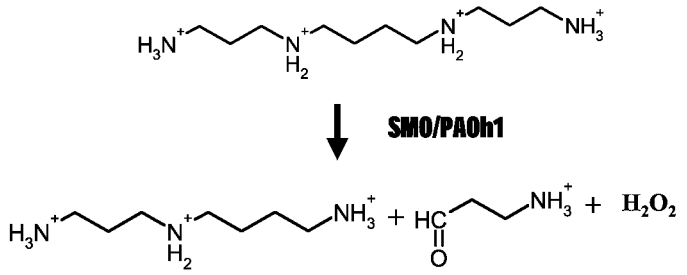


Fig. 8. SMO/PAOh1 activity. The FAD-dependent SMO/PAOh1 oxidize spermine-producing spermidine, 3-aminopropanal, and H_2O_2.

PAOh1 as the first human polyamine oxidase to be cloned) based on its homology to the maize plant polyamine oxidase *(125,133)*. Although it had all of the structural domains expected of an acetylpolyamine oxidase, which was the original target of our work, it demonstrated a very high affinity for, and oxidase activity with, spermine as a substrate. Importantly, it was also highly inducible, which set it apart from what was expected for the authentic PAO *(125)*. Vujcic et al. confirmed that this newly cloned enzyme was, in fact, a spermine oxidase, which they named SMO *(128)*. The human *SMO/PAOh1* gene is located on chromosome 20p13. Interestingly, this gene codes for multiple splice variants, with the longest open reading frame described thus far for the human gene being 1668 base pairs coding for a 555 amino acid protein with a predicted molecular weight of 61 kD *(125,134)*. The mouse and the human SMO/PAOh1 share considerable homology (95% identity) and substrate specificity *(128,135)*. The mouse gene also codes for multiple splice variants, one of which, mSMOm, is catalytically active and appears to be translocated to the nucleus *(136)*. It is also significant to note that a recently reported nuclear lysine demethylase (LSD1), which is part of a transcriptional corepressor complex, is highly homologous to human spermine oxidase *(137,138)*. Although LSD1 does not appear to use polyamines as substrates, the primary sequence similarity of this FAD-dependent methylase to SMO/PAOh1 strongly suggests that polyamines may interfere with its demethylase reaction, and thereby alter the repressor activity of the demethylase.

In human lung cancer cells, the induction of spermine oxidase in response to various polyamine analogs appears to occur primarily at the level of increased message. This increase appears to result from a combination of modestly increased transcription and a near doubling of mRNA half-life *(139)*. Significant posttranslational regulation of human SMO/PAOh1 has not been described.

Recombinant proteins of both the human and the mouse spermine oxidases have been characterized *(126,135)*, and both have very similar properties. The human protein exhibits a K_m of 1.6 m*M* for spermine and a V_{max} of 7.72 mmol/mg protein/min (k_{cat} = 7.2 s^{-1}) *(126)*. These values are somewhat different than those reported for the mouse homolog, in which the K_m and k_{cat} were 90–170 m*M* and 4.5–4.8 s^{-1}, respectively *(135,136,140)*. The basis for the apparent differences in kinetic constants of these very

similar homologs is not readily apparent; however, it should be stressed that the methods for preparation of the mouse and human proteins, and the assay systems used to measure the kinetic parameters, were different. The most significant difference was in the preparation of the human protein, where we purified a his-tagged recombinant protein from bacterial inclusion bodies by a denaturation/renaturation protocol *(126)*. In contrast, Mariottini, Federico, and colleagues purified native recombinant protein from periplasmically targeted constructs *(135,136,140)*. Importantly, multiple studies have demonstrated that the affinity and specificity of both mammalian enzymes are sufficient to be highly active *in situ*, and in specific cases, capable of reducing spermine concentrations to below detectable levels *(113,125,127,128)*.

The discovery of this enzyme underscores its potential to alter the response of both normal and tumor cells to the effects of various agents. This is particularly true for the potential effects SMO/PAOh1 could have on specific antitumor polyamine analogs. Our original discovery that human non-small-cell lung cancers respond to specific analog treatment with rapid and significant increases in both SMO/PAOh1 mRNA and activity, suggested a direct mechanism by which SMO/PAOh1 could alter cellular response to these agents *(125,127)*. Specifically, these results suggested that in addition to H_2O_2 resulting from the SSAT/PAO pathway, H_2O_2 produced by the direct oxidation of spermine could, in part, lead to the observed apoptotic response of various tumor cells to specific analogs. We originally proposed the production of H_2O_2 by polyamine catabolism downstream from SSAT activity as a contributing factor to polyamine analog-induced apoptosis; this hypothesis has been corroborated in subsequent studies *(99,100)*. However, these studies were completed prior to the discovery of SMO/PAOh1 and used MDL 72,527, which inhibits both PAO and SMO/PAOh1 to demonstrate the role of polyamine catabolism in cytotoxicity. Therefore, the origin of the toxic H_2O_2 is not clear *(99,100,113,126,128)*. An additional issue complicating the interpretation of the origin of H_2O_2 in analog-induced cells is that most analogs that induce SMO/PAOh1 also induce SSAT. To determine the contribution to cellular response by each of these pathways, double knockdown studies using RNA interference targeting either SSAT or SMO/PAOh1, and targeting both SSAT and SMO/PAOh1, are underway in our laboratory. Preliminary results strongly suggest that both pathways contribute to cytotoxicity and that reducing either SSAT or SMO/PAOh1 expression results in decreased sensitivity to specific analogs.

It is interesting that unlike PAO, SMO/PAOh1 does not effectively oxidize any of the terminally *bis*-alkylated polyamine analogs examined thus far *(115,126)*. However, N^1-monomethylspermine was determined to be a suitable substrate for SMO/PAOh1 *(128)*. Additionally, many of the analogs that were found to be substrates for PAO, are effective inhibitors of SMO/PAOh1 *(115,126)*. Taken together, these results suggest that analog structure must be critically considered if the goal is to induce cytotoxicity through SMO/PAOh1-produced H_2O_2.

5.2. Polyamine Catabolism as a Mediator of Cellular Damage and Its Implications in Disease Etiology

The likely link between increased spermine oxidation resulting in the production of H_2O_2 and its cellular response to specific polyamine analogs is currently under study in

several laboratories. How else might normal or dysregulated polyamine catabolism affect cells and organisms? Parchment and Pierce suggested that polyamine oxidation was important for normal embryonic development by producing the H_2O_2 necessary to kill unwanted cells as the embryo develops *(141,142)*. Although such an activation of polyamine catabolism in development would be beneficial to the organism, the possibility of detrimental activation of polyamine catabolism also exists.

One pathogenic state that leads to the inappropriate expression of spermine oxidase is *Helicobacter pylori* infection. *H. pylori* colonizes the mammalian stomach and is associated with gastritis, peptic ulcers, and gastric cancer. One question that persists regarding *H. pylori* infections is: how do the bacteria escape immune eradication? We have recently determined that infection with *H. pylori* results in a rapid induction of SMO/PAOh1 within the affected macrophages *(143)*. The observed induction of the oxidase produces sufficient H_2O_2 to lead to macrophage mitochondrial depolarization and apoptotic cell death. These data are entirely consistent with the hypothesis that *H. pylori*-induced SMO/PAOh1 and subsequent macrophage death play a significant role in the bacterium's ability to escape host immune defenses.

In addition to demonstrating a plausible mechanism for allowing persistent *H. pylori* infection, these results also indicate the possibility that mammalian cells other than macrophages may have their expression of polyamine catabolic enzymes altered by infection. *H. pylori* infection has been associated with gastric cancer, and oxidative stress is directly linked to carcinogenesis from oxidative damage of DNA. Therefore, based on the observations in *H. pylori*-infected macrophages, we sought to determine if similar effects would result in gastric epithelial cells exposed to *H. pylori* infection. We hypothesized that if *H. pylori* infection of gastric epithelial cells (the cells from which gastric cancers originate) results in sufficient spermine oxidase activity and H_2O_2 production to damage DNA and induce apoptosis, it is possible that this DNA damage will contribute to the genetic mutations necessary for *H. pylori*-induced carcinogenic transformation (Fig. 9). The results of these studies clearly demonstrate that exposure of human gastric epithelial cells to *H. pylori* results in increased *SMO/PAOh1* transcription, oxidase activity, DNA damage (as measured by increased 8-OH deoxyguanosine production), and apoptosis. Importantly, inhibition of the oxidase activity by MDL 72,527 or knockdown of *SMO/PAOh1* with specific small interfering RNAs, prevented the *H. pylori*-induced effects. In vivo results from both human and mouse tissues revealed that *H. pylori*-infected gastritis tissues demonstrated increased spermine oxidase expression, and when infection was eradicated in humans by antibiotic treatment, there was a concurrent decrease in *SMO/PAOh1* expression. Taken together, these results strongly implicate that *H. pylori*-induced SMO/PAOh1 activity in gastric epithelial cells plays an integral role in the disease process, and because of attendant oxidative DNA damage, may also be responsible for some of the genetic changes necessary for carcinogenic transformation in the etiology of gastric cancer.

Several questions remain to be answered regarding the potential for infectious agents to upregulate SMO/PAOh1 and potentially produce carcinogenic levels of reactive oxygen species in the form of H_2O_2. It is not known whether the ability to induce SMO/PAOh1 is limited to *H. pylori* infection or if other enteric bacterial infections

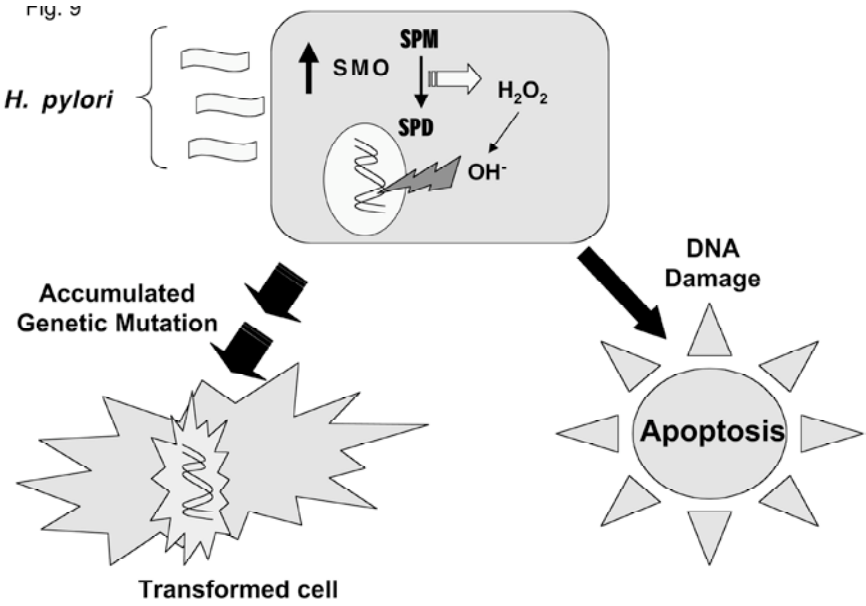


Fig. 9. Proposed model for *Helicobacter pylori*-induced transformation of gastric epithelial cells. *H. pylori* infection results in an increase in SMO/PAOh1 mRNA, protein, and oxidase activity. The resulting H_2O_2 damages DNA, which, if the damage is sufficient, can cause apoptosis or has the potential to lead to genetic mutation. If such mutations produce a growth or survival advantage, the potential for neoplastic transformation exists.

have similar effects. Chu et al. have demonstrated that glutathione peroxidase 1 and 2 double knockout mice have an increased incidence of microflora-associated intestinal cancers that correlate with increased inflammation subsequent to peroxidative stress *(144)*. If it is ultimately found that aberrant SMO/PAOh1 expression is directly associated with carcinogenic transformation, it would certainly point to the oxidase as a target for chemopreventive therapy.

6. Summary

The last few years have seen a dramatic increase in studies enhancing our knowledge of the regulation, function, and importance of the polyamine catabolic pathways. Instead of a single mammalian catabolic pathway regulated by SSAT, there exist at least two pathways by which spermine can be catabolized: the classical SSAT/PAO pathway and the direct catalysis of spermine by SMO/PAOh1. Although the precise interplay between these two pathways remains to be elucidated, clearly they are critical to the maintenance of normal polyamine homeostasis and have the potential to be dysregulated in various disease states. The emphasis of our group and others has been to understand the role that polyamine catabolism plays in the response of cells to the antitumor polyamine analogs, thus improving on our ability to target these pathways for therapeutic advantage. As is evident from the studies

cited, a considerable body of work has amassed toward the goal of understanding the molecular mechanisms by which the major players in polyamine catabolism are regulated.

It is now clear that induction of polyamine catabolism by specific analogs directly contributes to the cellular response caused by those agents, and that the response can be both cell- and tumor type-specific. The search for agents to exploit these responses is both active and ongoing. The recent finding demonstrating that increased SMO/PAOh1 activity may be involved in the etiology of a cancer of epithelial origin opens up an entirely new area that may lead to effective chemopreventive strategies. With a strong foundation of data on which to build, the coming years mark exciting times for both the study and exploitation of polyamine catabolism for disease intervention.

References

1. Metcalf, B. W., Bey, P., Danzin, C., Jung, M. J., Casara, P., and Vevert, J. P. (1978) Catalytic irreversible inhibition of mammalian ornithine decarboxylase (E. C4.1.1.17) by substrate and product analogues. *J. Am. Chem. Soc.* **100,** 2551–2553.
2. Gerner, E. W. and Meyskens, F. L., Jr. (2004) Polyamines and cancer: old molecules, new understanding. *Nat. Rev. Cancer* **4,** 781–792.
3. Bacchi, C. J. and Yarlett, N. (1993) Effects of antagonists of polyamine metabolism on African trypanosomes. *Acta Trop.* **54,** 225–236.
4. Bergeron, R. J., McManis, J. S., Liu, C. Z., et al. (1994) Antiproliferative properties of polyamine analogues: A structure-activity study. *J. Med. Chem.* **37,** 3464–3476.
5. Bergeron, R. J., Neims, A. H., McManis, J. S., et al. (1988) Synthetic polyamine analogues as antineoplastics. *J. Med. Chem.* **31,** 1183–1190.
6. Casero, R. A. and Woster, P. M. (2001) Terminally alkylated polyamine analogues as chemotherapeutic agents. *J. Med. Chem.* **44,** 1–26.
7. Thomas, T. and Thomas, T. J. (2001) Polyamines in cell growth and cell death: molecular mechanisms and therapeutic applications. *Cell Mol. Life Sci.* **58,** 244–258.
8. Frydman, B., Blokhin, A. V., Brummel, S., et al. (2003) Cyclopropane-containing polyamine analogues are efficient growth inhibitors of a human prostate tumor xenograft in nude mice. *J. Med. Chem.* **46,** 4586–4600.
9. Huang, Y., Hager, E. R., Phillips, D. L., et al. (2003) A novel polyamine analog inhibits growth and induces apoptosis in human breast cancer cells. *Clin. Cancer Res.* **9,** 2769–2777.
10. Huang, Y., Hager, E. R., Phillips, D. L., et al. (2002) Conformationally constrained polyamine analogues and oligoamines inhibit growth and induce apoptosis in human breast cancer cells. *Prof. Am. Assoc. Cancer Res.* **43,** 90.
11. Wallace, H. M. and Fraser, A. V. (2003) Polyamine analogues as anticancer drugs. *Biochem. Soc. Trans.* **31,** 393–396.
12. Fraser, A. V., Woster, P. M., Wallace, H. M. (2002) Induction of apoptosis in human leukaemic cells by IPENSpm, a novel polyamine analogue and anti-metabolite. *Biochem. J.* **367,** 307–312.
13. Casero, R. A., Jr., Celano, P., Ervin, S. J., Wiest, L., and Pegg, A. E. (1990) High specific induction of spermidine/spermine N^1-acetyltransferase in a human large cell lung carcinoma. *Biochem. J.* **270,** 615–620.
14. Casero, R. A., Jr., Celano, P., Ervin, S. J., Porter, C. W., Bergeron, R. J., and Libby, P. R. (1989) Differential induction of spermidine/spermine N^1-acetyltransferase in human lung cancer cells by the bis(ethyl)polyamine analogues. *Cancer Res.* **49,** 3829–3833.

15. Casero, R. A., Jr., Mank, A. R., Xiao, L., Smith, J., Bergeron, R. J., and Celano, P. (1992) Steady-state messenger RNA and activity correlates with sensitivity to N^1,N^{12}-bis(ethyl)spermine in human cell lines representing the major forms of lung cancer. *Cancer Res.* **52,** 5359–5363.
16. Casero, R. A., Wang, Y., Stewart, T. M., et al. (2003) The role of polyamine catabolism in anti-tumour drug response. *Biochem. Soc. Trans.* **31,** 361–365.
17. Seiler, N. (2004) Catabolism of polyamines. *Amino Acids* **26,** 217–233.
18. Wallace, H. M., Duthie, J., Evans, D. M., Lamond, S., Nicoll, K. M., and Heys, S. D. (2000) Alterations in polyamine catabolic enzymes in human breast cancer tissue. *Clin. Cancer Res.* **6,** 3657–3661.
19. Casero, R. A., Jr. and Pegg, A. E. (1993) Spermidine/spermine N^1-acetyltransferase—the turning point in polyamine metabolism. *FASEB J.* **7,** 653–661.
20. Casero, R. A., Jr., Celano, P., Ervin, S. J., Applegren, N. B., Wiest, L., and Pegg, A. E. (1991) Isolation and characterization of a cDNA clone that codes for human spermidine/spermine N^1-acetyltransferase. *J. Biol. Chem.* **266,** 810–814.
21. Pegg, A. E., Stanley, B. A., Wiest, L., and Casero, R. A., Jr. (1992) Nucleotide sequence of hamster spermidine/spermine-N^1-acetyltransferase cDNA. *Biochim. Biophys. Acta* **1171,** 106–108.
22. Fogel-Petrovic, M., Kramer, D. L., Ganis, B., Casero, R. A., Jr., and Porter, C. W. (1993) Cloning and sequence analysis of the gene and cDNA encoding mouse spermidine/spermine N^1-acetyltransferase—a gene uniquely regulated by polyamines and their analogs. *Biochim. Biophys. Acta* **1216,** 255–264.
23. Xiao, L., Celano, P., Mank, A. R., Pegg, A. E., and Casero, R. A., Jr. (1991) Characterization of a full-length cDNA which codes for the human spermidine/spermine N^1-acetyltransferase. *Biochem. Biophys. Res. Commun.* **179,** 407–415.
24. Xiao, L., Celano, P., Mank, A. R., et al. (1992) Structure of the human spermidine/spermine N^1-acetyltransferase gene (exon/intron gene organization and localization to Xp22.1). *Biochem. Biophys. Res. Commun.* **187,** 1493–1502.
25. Coleman, C. S., Huang, H., and Pegg, A. E. (1996) Structure and critical residues at the active site of spermidine/spermine-N^1-acetyltransferase. *Biochem. J.* **316,** 697–701.
26. Nikiforova, N. N., Velikodvorskaja, T. V., Kachko, A. V., et al. (2002) Induction of alternatively spliced spermidine/spermine N^1-acetyltransferase mRNA in the human kidney cells infected by venezuelan equine encephalitis and tick-borne encephalitis viruses. *Virology* **297,** 163–171.
27. Mita, K., Fukuchi, K., Hamana, K., Ichimura, S., and Nenoi, M. (2004) Accumulation of spermidine/spermine N^1-acetyltransferase and alternatively spliced mRNAs as a delayed response of HeLa S3 cells following X-ray irradiation. *Int. J. Radiat. Biol.* **80,** 369–375.
28. Fogel-Petrovic, M., Shappell, N. W., Bergeron, R. J., and Porter, C. W. (1993) Polyamine and polyamine analog regulation of spermidine/spermine N^1-acetyltransferase in MALME-3M human melanoma cells. *J. Biol. Chem.* **268,** 19,118–19,125.
29. Xiao, L. and Casero, R. A., Jr. (1996) Differential transcription of the human spermidine/spermine N^1-acetyltransferase (SSAT) gene in human lung carcinoma cells. *Biochem. J.* **313,** 691–696.
30. Fogel-Petrovic, M., Vujcic, S., Miller, J., and Porter, C. W. (1996) Differential post-transcriptional control of ornithine decarboxylase and spermidine-spermine N^1-acetyltransferase by polyamines. *FEBS Lett.* **391,** 89–94.

31. Parry, L., Balana Fouce, R., and Pegg, A. E. (1995) Post-transcriptional regulation of the content of spermidine/spermine N^1-acetyltransferase by N^1,N^{12}-bis(ethyl)spermine. *Biochem. J.* **305,** 451–458.
32. Coleman, C. S., Huang, H., and Pegg, A. E. (1995) Role of the carboxyl terminal MATEE sequence of spermidine/spermine N^1- acetyltransferase in the activity and stabilization by the polyamine analog N^1,N^{12}-bis(ethyl)spermine. *Biochemistry* **34,** 13,423–13,430.
33. Coleman, C. S. and Pegg, A. E. (1997) Proteasomal degradation of spermidine/spermine N^1-acetyltransferase requires the carboxyl-terminal glutamic acid residues. *J. Biol. Chem.* **272,** 12,164–12,169.
34. Rogers, S., Wells, R., and Rechsteiner, M. (1986) Amino acid sequences common to rapidly degraded proteins: The PEST hypothesis. *Science* **234,** 364–368.
35. Coleman, C. S. and Pegg, A. E. (2001) Polyamine analogues inhibit the ubiquitination of spermidine/spermine N^1-acetyltransferase and prevent its targeting to the proteasome for degradation. *Biochem. J.* **358,** 137–145.
36. Lu, L., Berkey, K. A., and Casero, R. A., Jr. (1996) RGFGIGS is an amino acid sequence required for acetyl coenzyme A binding and activity of human spermidine/spermine N^1-acetyltransferase. *J. Biol. Chem.* **271,** 18,920–18,924.
37. Wang, Y., Xiao, L., Thiagalingam, A., Nelkin, B. D., and Casero, R. A., Jr. (1998) The identification of a cis-element and a trans-acting factor involved in the response to polyamines and polyamine analogues in the regulation of the human spermidine/spermine N^1-acetyltransferase gene transcription. *J. Biol. Chem.* **273,** 34,623–34,630.
38. Wang, Y., Devereux, W., Stewart, T. M., and Casero, R. A., Jr. (1999) Cloning and characterization of human polyamine-modulated factor-1, a transcriptional cofactor that regulates the transcription of the spermidine/spermine N^1-acetyltransferase gene. *J. Biol. Chem.* **274,** 22,095–22,101.
39. Motohashi, H., Katsuoka, F., Engel, J. D., and Yamamoto, M. (2004) Small Maf proteins serve as transcriptional cofactors for keratinocyte differentiation in the Keap1-Nrf2 regulatory pathway. *Proc. Natl. Acad. Sci. USA* **101,** 6379–6384.
40. Kuroha, T., Takahashi, S., Komeno, T., Itoh, K., Nagasawa, T., and Yamamoto, M. (1998) Ablation of Nrf2 function does not increase the erythroid or megakaryocytic cell lineage dysfunction caused by p45 NF-E2 gene disruption. *J. Biochem. (Tokyo)* **123,** 376–379.
41. Itoh, K., Chiba, T., Takahashi, S., et al. (1997) An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem. Biophys. Res. Commun.* **236,** 313–322.
42. Marini, M. G., Chan, K., Casula, L., Kan, Y. W., Cao, A., and Moi, P. (1997) hMAF, a small human transcription factor that heterodimerizes specifically with Nrf1 and Nrf2. *J. Biol. Chem.* **272,** 16,490–16,497.
43. Wang, Y., Devereux, W., Stewart, T. M., and Casero, R. A., Jr. (2002) Polyamine-modulated factor 1 binds to the human homologue of the 7a subunit of the Arabidopsis COP9 signalosome: Implications in gene expression. *Biochem. J.* **366,** 79–86.
44. Porter, C. W., Ganis, B., Libby, P. R., and Bergeron, R. J. (1991) Correlations between polyamine analogue-induced increases in spermidine/spermine N^1-acetyltransferase activity, polyamine pool depletion, and growth inhibition in human melanoma cell lines. *Cancer Res.* **51,** 3715–3720.
45. Porter, C. W., Ganis, B., Rustum, Y., Wrzosek, C., Kramer, D. L., and Bergeron, R. J. (1994) Collateral sensitivity of human melanoma multidrug-resistant variants to the polyamine analogue, N^1,N^{11}-diethylnorspermine. *Cancer Res.* **54,** 5917–5924.

46. Chang, B. K., Bergeron, R. J., Porter, C. W., and Liang, Y. (1992) Antitumor effects of N-alkylated polyamine analogues in human pancreatic adenocarcinoma models. *Cancer Chemother. Pharmacol.* **30,** 179–182.
47. Chang, B. K., Bergeron, R. J., Porter, C. W., Vinson, J. R., Liang, Y., and Libby, P. R. (1992) Regulatory and antiproliferative effects of N-alkylated polyamine analogues in human and hamster pancreatic adenocarcinoma cell lines. *Cancer Chemother. Pharmacol.* **30,** 183–188.
48. Chang, B. K., Liang, Y., Miller, D. W., Bergeron, R. J., Porter, C. W., and Wang, G. (1993) Effects of diethyl spermine analogues in human bladder cancer cell lines in culture. *J. Urol.* **150,** 1293–1297.
49. McCloskey, D. E., Casero, R. A., Jr., Woster, P. M., and Davidson, N. E. (1995) Induction of programmed cell death in human breast cancer cells by an unsymmetrically alkylated polyamine analogue. *Cancer Res.* **55,** 3233–3236.
50. McCloskey, D. E., Yang, J., Woster, P. M., Davidson, N. E., and Casero, R. A., Jr. (1996) Polyamine analogue induction of programmed cell death in human lung tumor cells. *Clin. Cancer Res.* **2,** 441–446.
51. Bernacki, R. J., Bergeron, R. J., and Porter, C. W. (1992) Antitumor activity of N,N¢-bis(ethyl)spermine homologues against human MALME-3 melanoma xenografts. *Cancer Res.* **52,** 2424–2430.
52. Bernacki, R. J., Oberman, E. J., Seweryniak, K. E., Atwood, A., Bergeron, R. J., and Porter, C. W. (1995) Preclinical antitumor efficacy of the polyamine analogue N^1, N^{11}- diethylnorspermine administered by multiple injection or continuous infusion. *Clin. Cancer Res.* **1,** 847–857.
53. Porter, C. W., Bernacki, R. J., Miller, J., and Bergeron, R. J. (1993) Antitumor activity of N^1,N^{11}-bis(ethyl)norspermine against human melanoma xenografts and possible biochemical correlates of drug action. *Cancer Res.* **53,** 581–586.
54. Casero, R. A., Jr., Gabrielson, E. W., and Pegg, A. E. (1994) Immunohistochemical staining of human spermidine/spermine N^1-acetyltransferase superinduced in response to treatment with antitumor polyamine analogues. *Cancer Res.* **54,** 3955–3958.
55. Gabrielson, E. W., Pegg, A. E., and Casero, R. A., Jr. (1999) The induction of spermidine/spermine N^1-acetyltransferase (SSAT) is a common event in the response of human primary non-small cell lung carcinomas to exposure to the new antitumor polyamine analogue N^1,N^{11}-bis(ethyl)norspermine. *Clin. Cancer Res.* **5,** 1638–1641.
56. Shah, N., Antony, T., Haddad, S., et al. (1999) Antitumor effects of bis(ethyl)polyamine analogs on mammary tumor development in FVB/NTgN (MMTVneu) transgenic mice. *Cancer Lett.* **146,** 15–23.
57. Gabrielson, E., Tully, E., Hacker, A., Pegg, A. E., Davidson, N. E., and Casero, R. A., Jr. (2004) Induction of spermidine/spermine N^1-acetyltransferase in breast cancer tissues treated with the polyamine analogue N^1, N^{11}-diethylnorspermine. *Cancer Chemother. Pharmacol.* **54,** 122–126.
58. Mank-Seymour, A. R., Murray, T. R., Berkey, K. A., Xiao, L., Kern, S., Casero, R. A., Jr. (1998) Two active copies of the X-linked gene spermidine/spermine N^1- acetyltransferase (SSAT) in a female lung cancer cell line are associated with an increase in sensitivity to an antitumor polyamine analogue. *Clin. Cancer Res.* **4,** 2003–2008.
59. Parry, L., Lopez-Ballester, J., Wiest, L., and Pegg, A. E. (1995) Effect of expression of human spermidine/spermine N^1-acetyltransferase in *Escherichia coli*. *Biochemistry* **34,** 2701–2709.

60. Vujcic, S., Halmekyto, M., Diegelman, P., et al. (2000) Effects of conditional overexpression of spermidine/spermine N^1- acetyltransferase on polyamine pool dynamics, cell growth, and sensitivity to polyamine analogs. *J. Biol. Chem.* **275,** 38,319–38,328.
61. Murray-Stewart, T., Applegren, N. B., Devereux, W., et al. (2003) Spermidine/spermine N^1-acetyltransferase (SSAT) activity in human small-cell lung carcinoma cells following transfection with a genomic SSAT construct. *Biochem. J.* **373,** 629–634.
62. McCloskey, D. E. and Pegg, A. E. (2003) Properties of the spermidine/spermine N^1-acetyltransferase mutant L156F that decreases cellular sensitivity to the polyamine analogue N^1, N^{11}-bis(ethyl)norspermine. *J. Biol. Chem.* **278,** 13,881–13,887.
63. McCloskey, D. E. and Pegg, A. E. (2000) Altered spermidine/spermine N^1-acetyltransferase activity as a mechanism of cellular resistance to bis(ethyl)polyamine analogues. *J. Biol. Chem.* **275,** 28,708–28,714.
64. Chen, Y., Kramer, D. L., Jell, J., Vujcic, S., and Porter, C. W. (2003) Small interfering RNA suppression of polyamine analog-induced spermidine/spermine N^1-acetyltransferase. *Mol. Pharmacol.* **64,** 1153–1159.
65. Chen, Y., Vujcic, S., Liang, P., Diegelman, P., Kramer, D. L., and Porter, C. W. (2003) Genomic identification and biochemical characterization of a second spermidine/spermine N^1-acetyltransferase. *Biochem. J.* **373,** 661–667.
66. Coleman, C. S., Stanley, B. A., Jones, A. D., and Pegg, A. E. (2004) Spermidine/spermine N^1-acetyltransferase-2 (SSAT2) acetylates thialysine and is not involved in polyamine metabolism. *Biochem. J.* **384,** 139–148.
67. Alhonen, L., Karppinen, A., Uusi-Oukari, M., et al. (1998) Correlation of polyamine and growth responses to N^1,N^{11}- diethylnorspermine in primary fetal fibroblasts derived from transgenic mice overexpressing spermidine/spermine N^1-acetyltransferase. *J. Biol. Chem.* **273,** 1964–1969.
68. Alhonen, L., Parkkinen, J. J., Keinanen, T., Sinervirta, R., Herzig, K. H., and Janne, J. (2000) Activation of polyamine catabolism in transgenic rats induces acute pancreatitis. *Proc. Natl. Acad. Sci. USA* **97,** 8290–8295.
69. Alhonen, L., Pietila, M., Halmekyto, M., Kramer, D. L., Janne, J., and Porter, C. W. (1999) Transgenic mice with activated polyamine catabolism due to overexpression of spermidine/spermine N^1-acetyltransferase show enhanced sensitivity to the polyamine analog, N^1, N^{11}- diethylnorspermine. *Mol. Pharmacol.* **55,** 693–698.
70. Alhonen, L., Rasanen, T. L., Sinervirta, R., et al. (2002) Polyamines are required for the initiation of rat liver regeneration. *Biochem. J.* **362,** 149–153.
71. Bras, A. P., Janne, J., Porter, C. W., and Sitar, D. S. (2001) Spermidine/spermine N^1-acetyltransferase catalyzes amantadine acetylation. *Drug Metab. Dispos.* **29,** 676–680.
72. Coleman, C. S., Pegg, A. E., Megosh, L. C., Guo, Y., Sawicki, J. A., and O'Brien, T. G. (2002) Targeted expression of spermidine/spermine N^1-acetyltransferase increases susceptibility to chemically induced skin carcinogenesis. *Carcinogenesis* **23,** 359–364.
73. Kaasinen, K., Koistinaho, J., Alhonen, L., and Janne, J. (2000) Overexpression of spermidine/spermine N^1-acetyltransferase in transgenic mice protects the animals from kainate-induced toxicity. *Eur. J. Neurosci.* **12,** 540–548.
74. Kaasinen, S. K., Grohn, O. H., Keinanen, T. A., Alhonen, L., and Janne, J. (2003) Overexpression of spermidine/spermine N^1-acetyltransferase elevates the threshold to pentylenetetrazol-induced seizure activity in transgenic mice. *Exp. Neurol.* **183,** 645–652.
75. Kaasinen, S. K., Oksman, M., Alhonen, L., Tanila, H., and Janne, J. (2004) Spermidine/spermine N^1-acetyltransferase overexpression in mice induces hypoactivity and spatial learning impairment. *Pharmacol. Biochem. Behav.* **78,** 35–45.

76. Kee, K., Foster, B. A., Merali, S., et al. (2004) Activated polyamine catabolism depletes acetyl-CoA pools and suppresses prostate tumor growth in TRAMP mice. *J. Biol. Chem.* **279,** 40,076–40,083.
77. Min, S. H., Simmen, R. C., Alhonen, L., et al. (2002) Altered levels of growth-related and novel gene transcripts in reproductive and other tissues of female mice overexpressing spermidine/spermine N^1-acetyltransferase (SSAT). *J. Biol. Chem.* **277,** 3647–3657.
78. Pegg, A. E., Feith, D. J., Fong, L. Y., Coleman, C. S., O'Brien, T. G., and Shantz, L. M. (2003) Transgenic mouse models for studies of the role of polyamines in normal, hypertrophic and neoplastic growth. *Biochem. Soc. Trans.* **31,** 356–360.
79. Rasanen, T. L., Alhonen, L., Sinervirta, R., et al. (2003) Gossypol activates pancreatic polyamine catabolism in normal rats and induces acute pancreatitis in transgenic rats overexpressing spermidine/spermine N^1-acetyltransferase. *Scand. J. Gastroenterol.* **38,** 787–793.
80. Suppola, S., Heikkinen, S., Parkkinen, J. .J, et al. (2001) Concurrent overexpression of ornithine decarboxylase and spermidine/spermine N^1-acetyltransferase further accelerates the catabolism of hepatic polyamines in transgenic mice. *Biochem. J.* **358,** 343–348.
81. Suppola, S., Pietila, M., Parkkinen, J. J., et al. (1999) Overexpression of spermidine/spermine N^1-acetyltransferase under the control of mouse metallothionein I promoter in transgenic mice: Evidence for a striking post-transcriptional regulation of transgene expression by a polyamine analogue. *Biochem. J.* **338,** 311–316.
82. Janne, J., Alhonen, L., Pietila, M., and Keinanen, T. A. (2004) Genetic approaches to the cellular functions of polyamines in mammals. *Eur. J. Biochem.* **271,** 877–894.
83. Kee, K., Vujcic, S., Merali, S., et al. (2004) Metabolic and antiproliferative consequences of activated polyamine catabolism in LNCaP prostate carcinoma cells. *J. Biol. Chem.* **279,** 27,050–27,058.
84. Babbar, N., Ignatenko, N. A., Casero, R. A., Jr., and Gerner, E. W. (2003) Cyclooxygenase-independent induction of apoptosis by sulindac sulfone is mediated by polyamines in colon cancer. *J. Biol. Chem.* **278,** 47,762–47,775.
85. Maxwell, P. J., Longley, D. B., Latif, T., et al. (2003) Identification of 5-fluorouracil-inducible target genes using cDNA microarray profiling. *Cancer Res.* **63,** 4602–4606.
86. Hector, S., Porter, C. W., Kramer, D. L., et al. (2004) Polyamine catabolism in platinum drug action: Interactions between oxaliplatin and the polyamine analogue N^1,N^{11}-diethylnorspermine at the level of spermidine/spermine N^1-acetyltransferase. *Mol. Cancer Ther.* **3,** 813–822.
87. Choi, W., Gerner, E. W., Ramdas, L., et al. (2005) Combination of 5-fluorouracil and N^1, N^{11}-diethylnorspermine markedly activates spermidine/spermine N^1-acetyltransferase expression, depletes polyamines, and synergistically induces apoptosis in colon carcinoma cells. *J. Biol. Chem.* **280,** 3295–3304
88. Hahm, H. A., Dunn, V. R., Butash, K. A., et al. (2001) Combination of standard cytotoxic agents with polyamine analogues in the treatment of breast cancer cell lines. *Clin. Cancer Res.* **7,** 391–399.
89. Matsui, I., Wiegand, L., Pegg, A. E. (1981) Properties of spermidine N^1-acetyltransferase from livers of rats treated with carbon tetrachloride and its role in the conversion of spermidine into putrescine. *J. Biol. Chem.* **256,** 2454–2459.
90. Della Ragione, F., and Pegg, A. E. (1983) Studies of the specificity and kinetics of rat liver spermidine/spermine N^1-acetyltransferase. *Biochem. J.* **213,** 701–706.
91. Ragione, F. D., and Pegg, A. E. (1982) Purification and characterization of spermidine/spermine N^1-acetyltransferase from rat liver. *Biochemistry* **21,** 6152–6158.

92. Nagesh Babu, G., Sailor, K. A., Sun, D., and Dempsey, R. J. (2001) Spermidine/spermine N^1-acetyl transferase activity in rat brain following transient focal cerebral ischemia and reperfusion. *Neurosci. Lett.* **300,** 17–20.
93. Weiss, T. S., Bernhardt, G., Buschauer, A., et al. (2002) Polyamine levels of human colorectal adenocarcinomas are correlated with tumor stage and grade. *Int. J. Colorectal Dis.* **17,** 381–387.
94. Adibhatla, R. M., Hatcher, J. F., Sailor, K., and Dempsey, R. J. (2002) Polyamines and central nervous system injury: Spermine and spermidine decrease following transient focal cerebral ischemia in spontaneously hypertensive rats. *Brain Res.* **938,** 81–86.
95. Rao, A. M., Hatcher, J. F., Dogan, A., and Dempsey, R. J. (2000) Elevated N^1-acetylspermidine levels in gerbil and rat brains after CNS injury. *J. Neurochem.* **74,** 1106–1111.
96. Zoli, M., Pedrazzi, P., Zini, I., and Agnati, L. F. (1996) Spermidine/spermine N^1-acetyltransferase mRNA levels show marked and region-specific changes in the early phase after transient forebrain ischemia. *Brain Res. Mol. Brain Res.* **38,** 122–134.
97. Zahedi, K., Wang, Z., Barone, S., et al. (2003) Expression of SSAT, a novel biomarker of tubular cell damage, increases in kidney ischemia-reperfusion injury. *Am. J. Physiol. Renal Physiol.* **284,** F1046–F1055.
98. Wang, Z., Zahedi, K., Barone, S., et al. (2004) Overexpression of SSAT in kidney cells recapitulates various phenotypic aspects of kidney ischemia-reperfusion injury. *J. Am. Soc. Nephrol.* **15,** 1844–1852.
99. Ha, H. C., Woster, P. M., Yager, J. D., and Casero, R. A., Jr. (1997) The role of polyamine catabolism in polyamine analogue-induced programmed cell death. *Proc. Natl. Acad. Sci. USA* **94,** 11,557–11,562.
100. Chen, Y., Kramer, D. L., Diegelman, P., Vujcic, S., and Porter, C. W. (2001) Apoptotic signaling in polyamine analogue-treated SK-MEL-28 human melanoma cells. *Cancer Res.* **61,** 6437–6444.
101. Holtta, E. (1977) Oxidation of spermidine and spermine in rat liver: purification and properties of polyamine oxidase. *Biochemistry* **16,** 91–100.
102. Bolkenius, F. N. and Seiler, N. (1981) Acetyl derivatives as intermediates in polyamine catabolism. *Int. J. Biochem.* **13,** 287–292.
103. Seiler, N., Bolkenius, F. N., and Rennert, O. M. (1981) Interconversion, catabolism and elimination of the polyamines. *Med. Biol.* **59,** 334–346.
104. Bolkenius, F. N. and Seiler, N. (1987) The role of polyamine reutilization in depletion of cellular stores of polyamines in non-proliferating tissues. *Biochim. Biophys. Acta* **923,** 125–135.
105. Seiler, N. (1987) Functions of polyamine acetylation. *Can. J. Physiol. Pharmacol.* **65,** 2024–2035.
106. Holtta, E. (1983) Polyamine oxidase (rat liver). *Meth. Enzymol.* **94,** 306–311.
107. Seiler, N. (1990) Polyamine metabolism. *Digestion* **46,** 319–330.
108. Wallace, H. M., Nuttall, M. E., and Coleman, C. S. (1988) Polyamine recycling enzymes in human cancer cells. *Adv. Exp. Med. Biol.* **250,** 331–344.
109. Seiler, N. (1995) Polyamine oxidase, properties and functions. *Prog. Brain Res.* **106,** 333–344.
110. Seiler, N. (2000) Oxidation of polyamines and brain injury. *Neurochem. Res.* **25,** 471–490.
111. Seiler, N., Duranton, B., and Raul, F. (2002) The polyamine oxidase inactivator MDL 72527. *Prog. Drug Res.* **59,** 1–40.
112. Morgan, D. M. (1998) Polyamine oxidases—enzymes of unknown function? *Biochem. Soc. Trans.* **26,** 586–591.

113. Vujcic, S., Liang, P., Diegelman, P., Kramer, D. L., Porter, C. W. (2003) Genomic identification and biochemical characterization of the mammalian polyamine oxidase involved in polyamine back-conversion. *Biochem. J.* **370,** 19–28.
114. Wu, T., Yankovskaya, V., and McIntire, W. S. (2003) Cloning, sequencing, and heterologous expression of the murine peroxisomal flavoprotein, N^1-acetylated polyamine oxidase. *J. Biol. Chem.* **278,** 20,514–20,525.
115. Wang, Y., Hacker, A., Murray-Stewart, T., et al. (2005) Properties of recombinant human N^1-acetylpolyamine oxidase (hPAO): potential role in determining drug sensitivity. *Cancer Chemother. Pharmacol.* **56,** 83–90.
116. Gould, S. J., Keller, G. A., Hosken, N., Wilkinson, J., and Subramani, S. (1989) A conserved tripeptide sorts proteins to peroxisomes. *J. Cell Biol.* **108,** 1657–1664.
117. Libby, P. R. and Porter, C. W. (1987) Separation of two isozymes of polyamine oxidase from murine L1210 leukemia cells. *Biochem. Biophys. Res. Commun.* **144,** 528–535.
118. Bergeron, R. J., Weimar, W. R., Luchetta, G., et al. (1995) Metabolism and pharmacokinetics of N^1,N^{11}-diethylnorspermine. *Drug Metab. Dispos.* **23,** 1117–1125.
119. Lawson, K. R., Marek, S., Linehan, J. A., et al. (2002) Detoxification of the polyamine analogue N^1-ethyl-N^{11}-[(cycloheptyl)methy]-4,8-diazaundecane (CHENSpm) by polyamine oxidase. *Clin. Cancer Res.* **8,** 1241–1247.
120. Bolkenius, F. N. and Seiler, N. (1989) New substrates of polyamine oxidase. Dealkylation of N-alkyl-alpha, omega-diamines. *Biol. Chem. Hoppe Seyler* **370,** 525–531.
121. Bitonti, A. J., Dumont, J. A., Bush, T. L., Stemerick, D. M., Edwards, M. L., and McCann, P. P. (1990) Bis(benzyl)polyamine analogs as novel substrates for polyamine oxidase. *J. Biol. Chem.* **265,** 382–388.
122. Seiler, N., Douaud, F., Renault, J., et al. (1998) Polyamine sulfonamides with NMDA antagonist properties are potent calmodulin antagonists and cytotoxic agents. *Int. J. Biochem. Cell Biol.* **30,** 393–406.
123. Bey, P., Bolkenius, F. N., Seiler, N., and Casara, P. (1985) N-2,3-Butadienyl-1,4-butanediamine derivatives: potent irreversible inactivators of mammalian polyamine oxidase. *J. Med. Chem.* **28,** 1–2.
124. Claverie, N., Wagner, J., Knodgen, B., and Seiler, N. (1987) Inhibition of polyamine oxidase improves the antitumoral effect of ornithine decarboxylase inhibitors. *Anticancer Res.* **7,** 765–772.
125. Wang, Y., Devereux, W., Woster, P. M., Stewart, T. M., Hacker, A., and Casero, R. A., Jr. (2001) Cloning and characterization of a human polyamine oxidase that is inducible by polyamine analogue exposure. *Cancer Res.* **61,** 5370–5373.
126. Wang, Y., Murray-Stewart, T., Devereux, W., et al. (2003) Properties of purified recombinant human polyamine oxidase, PAOh1/SMO. *Biochem. Biophys. Res. Commun.* **304,** 605–611.
127. Devereux, W., Wang, Y., Stewart, T. M., et al. (2003) Induction of the PAOh1/SMO polyamine oxidase by polyamine analogues in human lung carcinoma cells. *Cancer Chemother. Pharmacol.* **52,** 383–390.
128. Vujcic, S., Diegelman, P., Bacchi, C. J., Kramer, D. L., and Porter, C. W. (2002) Identification and characterization of a novel flavin-containing spermine oxidase of mammalian cell origin. *Biochem. J.* **367,** 665–675.
129. Mitchell, J.L., Leyser, A., Holtorff, M. S., et al. (2002) Antizyme induction by polyamine analogues as a factor in cell growth inhibition. *Biochem. J.* **366,** 663–671.

130. Reddy, V. K., Valasinas, A., Sarkar, A., Basu, H. S., Marton, L. J., and Frydman, B. (1998) Conformationally restricted analogues of N^1,N^{12}-bisethylspermine: synthesis and growth inhibitory effects on human tumor cell lines. *J. Med. Chem.* **41,** 4723–4732.
131. Reddy, V. K., Sarkar, A., Valasinas, A., Marton, L. J., Basu, H. S., and Frydman, B. (2001) cis-Unsaturated analogues of 3,8,13,18,23-pentaazapentacosane (BE-4-4-4-4): Synthesis and growth inhibitory effects on human prostate cancer cell lines. *J. Med. Chem.* **44,** 404–417.
132. Valasinas, A., Reddy, V. K., Blokhin, A. V., et al. (2003) Long-chain polyamines (oligoamines) exhibit strong cytotoxicities against human prostate cancer cells. *Bioorg. Med. Chem.* **11,** 4121–4131.
133. Tavladoraki, P., Schinina, M. E., Cecconi, F., et al. (1998) Maize polyamine oxidase: primary structure from protein and cDNA sequencing. *FEBS Lett.* **426,** 62–66.
134. Murray-Stewart, T., Wang, Y., Devereux, W., and Casero, R. A., Jr. (2002) Cloning and characterization of multiple human polyamine oxidase splice variants that code for isoenzymes with different biochemical characteristics. *Biochem. J.* **368,** 673–677.
135. Cervelli, M., Polticelli, F., Federico, R., and Mariottini, P. (2003) Heterologous expression and characterization of mouse spermine oxidase. *J. Biol. Chem.* **278,** 5271–5276.
136. Cervelli, M., Bellini, A., Bianchi, M., et al. (2004) Mouse spermine oxidase gene splice variants. Nuclear subcellular localization of a novel active isoform. *Eur. J. Biochem.* **271,** 760–770.
137. Shi, Y., Sawada, J., Sui, G., et al. (2003) Coordinated histone modifications mediated by a CtBP co-repressor complex. *Nature* **422,** 735–738.
138. Shi, Y., Lan, F., Matson, C., et al. (2004) Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* **119,** 941–953.
139. Wang, Y., Hacker, A., Murray-Stewart, T., Fleischer, J. G., Woster, P. M., and Casero, R. A. (2005) Induction of human spermine oxidase SMO(PAOh1) is regulated at the levels of new mRNA synthesis, mRNA stabilization, and newly synthesized protein. *Biochem. J.* **386,** 543–547.
140. Bellelli, A., Cavallo, S., Nicolini, L., et al. (2004) Mouse spermine oxidase: a model of the catalytic cycle and its inhibition by N,N^1-bis(2,3-butadienyl)-1,4-butanediamine. *Biochem. Biophys. Res. Commun.* **322,** 1–8.
141. Parchment, R. E., and Pierce, G. B. (1989) Polyamine oxidation, programmed cell death, and regulation of melanoma in the murine embryonic limb. *Cancer Res.* **49,** 6680–6686.
142. Parchment, R. E. (1993) The implications of a unified theory of programmed cell death, polyamines, oxyradicals and histogenesis in the embryo. *Int. J. Dev. Biol.* **37,** 75–83.
143. Chaturvedi, R., Cheng, Y., Asim, M., et al. (2004) Induction of polyamine oxidase 1 by Helicobacter pylori causes macrophage apoptosis by hydrogen peroxide release and mitochondrial membrane depolarization. *J. Biol. Chem.* **279,** 40,161–40,173.
144. Chu, F. F., Esworthy, R. S., Chu, P. G., et al. (2004) Bacteria-induced intestinal cancer in mice with disrupted Gpx1 and Gpx2 genes. *Cancer Res.* **64,** 962–968.

13

Cellular Signaling and Polyamines in the Control of Apoptosis in Intestinal Epithelial Cells

Leonard R. Johnson and Ramesh M. Ray

1. Introduction

A recent review article by Jänne et al. *(1)* begins with, "The polyamines, putrescine, spermidine, and spermine, are organic cations shown to participate in a bewildering number of cellular reactions, yet their exact functions in intermediary metabolism and specific interactions with cellular components remain largely elusive." As documented by Schipper et al. *(2)* in their article entitled "Involvement of polyamines in apoptosis. Facts and controversies: effectors or protectors?" apoptosis or programmed cell death certainly belongs on the list. As the title of the Schipper et al. review indicates, the role of polyamines in apoptosis is anything but clear. In most, but not all instances, ornithine decarboxylase (ODC) activity increased with the induction of apoptosis, but there was no clear cut change in polyamine levels as they increased, decreased, or stayed the same depending on the system *(2)*. Most experiments examining the effect of blocking ODC with α-difluoromethylornithine (DFMO) reported protection from apoptosis, whereas a few found no effect *(2)*. The effects of polyamine analogs on apoptosis were also variable *(2)*.

A number of explanations for the disparate results come to mind. The obvious ones are the use of different cell types and different inducers of apoptosis. Less obvious causes for discrepancies include different intervals for measuring apoptosis after drug administration and the failure to quantify polyamine levels. For example, was DFMO administered for a sufficient time interval to deplete polyamines or did it only inhibit ODC? In addition, almost no attention has been paid to where in the apoptotic process polyamines might be exerting effects.

In this chapter, we examine the effects of polyamine depletion caused by DFMO on apoptosis in a line of untransformed intestinal epithelial cells (IECs), paying particular attention to the alterations produced in signaling pathways that regulate apoptosis. Most experiments to be discussed were conducted using IEC-6 cells, a cell line originally developed by Quaroni et al. *(3)* from crypt cells of the rat small intestine. Under

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the conditions in which they are used in these studies, the cells remain undifferentiated. In most experiments, cells are incubated in DFMO for 4 d. We have shown that under these conditions IEC-6 cells are depleted of putrescine within 3 h. In fact, 50% of the putrescine is gone within 1 h. Spermidine is depleted within 24 h and spermine is reduced to 40% of control levels after 4 d *(4)*. All the effects of DFMO to be described are prevented if 10 μ*M* putrescine is added to the medium along with DFMO. Thus results are due entirely to polyamine depletion. We describe several instances in which DFMO produces effects as early as 1 h after administration. The cells still contain large amounts of polyamines, yet these early effects are also prevented by exogenous putrescine and are owing to polyamine depletion.

2. Apoptosis

Programmed cell death occurs via the process termed *apoptosis* and is the result of initiating a signal cascade that activates specific enzymes that dismantle the cell. The process can be initiated by cell damage, withdrawal of growth factors, and by the binding of ligands to cell membrane receptors of the tumor necrosis factor (TNF) family. Apoptosis usually begins with dense chromosome condensation at the periphery of the nucleus. The chromatin is compacted and segregated and the cell begins to shrink as the cytoplasm condenses. The nuclear fragments and the cell break up into membrane-bound apoptotic bodies, which are phagocytosed by other cells *(5)*. The defining feature of this process is that cellular contents are never released to produce inflammation, which is the result of cell death caused by necrosis.

2.1. Proteins Involved in Apoptosis

The general steps involved in apoptosis are remarkably conserved during the evolutionary process, and are due to three types of proteins. These may be labeled as effectors, accessory molecules or adaptors, and regulators. A family of cysteine proteases called caspases are the effectors of apoptosis *(6)*. Caspases exist in the cytoplasm as presynthesized proenzymes, and are activated in response to apoptotic stimuli. There are two basic types of caspases. Caspases 3, 6, and 7 are termed *executioner caspases* and dismantle the cell by cleaving specific proteins, such as lamin, various nuclear proteins, and cytoskeletal proteins. They also activate enzymes, such as caspase-activated DNAse and DNA fragmentation factor, which cleave DNA into fragments resulting in so-called laddering that is used as an assay to detect apoptosis *(7)*. Initiator caspases, such as caspase 8 and 9, are responsible for activating the executioner caspases.

Adaptor or accessory molecules are involved in the activation or inhibition of caspases. The most familiar of these is cytochrome c, which is released from mitochondria during the apoptotic process. Cytochrome c binds monomers of apoptotic protease-activating factor (APAF)-1, another adapter, in the cytosol to form the heptameric apoptosome. The apoptosome then recruits and binds procaspase 9, which is proteolytically cleaved to form active caspase 9. Caspase 9 then activates executioner caspases 3, 6, and 7 *(8)*.

Inhibitor of apoptosis proteins (IAPs) are capable of directly inhibiting activated caspases and probably act as insurance against spontaneous activation of caspases.

Synthesis of IAPs is also stimulated by transcription factors, such as nuclear transcription factor κB (NF-κB) and the extracellular signal-regulated kinase (ERKs) *(9)*. IAPs include XIAP, cIAP1, cIAP2, and survivin. IAP activity can be inhibited by additional accessory molecules released from mitochondria. These include second mitochondrial activator of caspase (SMAC) and high-temperature requirement service protease ($HtrA_2$) *(10)*.

The final group of molecules involved in apoptosis are the members of the Bcl-2 family of proteins, which regulate the release of cytochrome c from mitochondria and alter the ability of APAF-1 to activate caspases. A large number of Bcl-2 proteins has been identified and each possesses at least one of four conserved motifs known as Bcl-2 homology (BH) domains. Bcl-2 proteins are divided into three groups on the basis of their structures and functions. The first consists of antiapoptotic channel-forming proteins having four BH domains, a transmembrane anchoring sequence, and includes Bcl-2 itself, Bcl-X_L, and Bcl-w. The second group is proapoptotic channel-forming proteins with BH_1, BH_2, and BH_3 domains plus the transmembrane region, and includes Bax and Bak. The third group consists of proapoptotic proteins with only the BH_3 domain and includes Bad and Bid. They are often referred to as BH_3-only proteins and dimerize with the membrane anchored channel-forming Bcl-2 receptors *(11)*. Pro- and antiapoptotic members can form homo- and heterodimers. Whether a cell lives or dies may depend on the relative amounts of the two forms *(12)*. An excess of proapoptotic members will heterodimerize with all the antiapoptotic proteins, leaving homodimers of apoptotic proteins to form channels and release cytochrome c. An excess of prosurvival proteins prevents cytochrome c release and may also prevent the activation of caspase 9 *(12)*.

2.2. Apoptotic Pathways

Apoptosis is initiated by the activation of one or both of two major pathways. An extrinsic pathway (Fig. 1) is initiated by ligands, such as TNF-α and FasL, which, depending on cell type and circumstances, can result in growth arrest, apoptosis, or cell division *(13)*. Death receptors of the TNF family, such as TNFR1, Fas, and TRAIL, recruit, on binding ligand, molecules that make up a death-inducing complex that includes procaspase 8 *(14)*. This activates caspase 8, which in turn activates caspase 3, but also results in the activation of regulatory pathways, including those mediated by NF-κB , mitogen-activated protein kinases, and c-Jun NH_2-terminal kinases (JNKs) *(15–17)*. The activation of these additional pathways explains why TNF-α usually does not cause apoptosis unless an inhibitor of protein synthesis is present. When NF-κB is activated, it becomes a transcription factor for Bcl-2, Bcl-X_L, and various IAPs. Activation of JNK usually causes apoptosis, but in some cell lines, including IEC-6 cells, JNK is inhibited by the activation of MEK1 or ERK 1/2 *(18)*.

The second major pathway is the intrinsic or mitochondrial pathway, which results in the release of cytochrome c from mitochondria and the activation of caspase 9 (Fig. 2). This pathway is regulated by the Bcl-2 family proteins and is usually initiated by DNA damage. Agents such as chemotherapeutic drugs, ultraviolet irradiation, γ-irradiation, and reactive oxygen species frequently activate p53, which as a transcription factor leads to the synthesis of proapoptotic members of the Bcl-2 family, such as Bax and

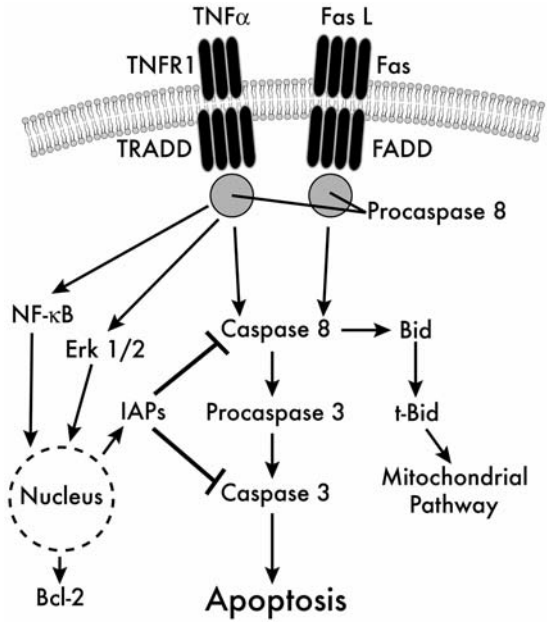

Fig. 1. Extrinsic or death receptor pathway. (*See* Subheading 2.2. for explanation and discussion.)

Bak. There is also crosstalk between the extrinsic and intrinsic pathways, for caspase 8 cleaves Bid forming the active, truncated *t*-Bid, which can bind Bcl-2 and inactivate it, resulting in apoptosis.

3. Intestinal Cells as Models for Apoptosis

The intestinal epithelium provides an interesting, yet complicated, model to study apoptosis. This tissue has one of the most rapid turnover rates known because cells lost to the lumen are replaced by the progeny of stem cells (Fig. 3). After division of the stem cells, one daughter cell remains anchored as the new stem cell, whereas the other undergoes several divisions in the lower and middle thirds of the crypt. Extra stem cells are eliminated by a process of spontaneous apoptosis. Each crypt produces 13–16 cells/h *(19)*. Most of these migrate out of the crypt and onto a villus where they continue to migrate toward the villus tip. During migration, the cells mature into three of the four terminally differentiated cell types of the adult small intestinal epithelium: the absorptive enterocyte, the enteroendocrine cell, and the mucus-secreting goblet cell. A few cells migrate to the base of the crypt to become lysozyme-producing Paneth cells. Cells that migrate onto a villus are eventually shed by a process that is incompletely understood. Shedding involves apoptosis, and the question is whether the apoptosis occurs on the villus or whether it is triggered by detachment from the substrate, a process known as anoikis *(20)*. The whole process from proliferation to shedding occurs in only 2–3 d *(19)*. Apoptosis of dividing crypt cells can be induced by damaging agents and is dependent on the activation of Bax.

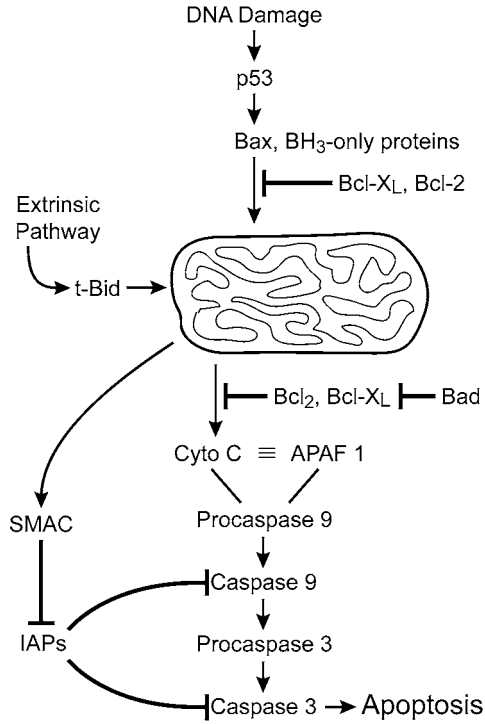

Fig. 2. Intrinsic or mitochondrial pathway. (*See* Subheading 2.2. for explanation and discussion.)

The intestinal epithelium of the mouse has been the primary model for studying gut apoptosis. Many of these have identified the roles of members of the Bcl-2 family of proteins and p53. Little is known of the overall regulatory process of apoptosis in the intestine, and there has been little progress in developing appropriate in vitro models of normal intestinal cells. The most widely used intestinal cell lines have been derived from tumors and are abnormal. Some, such as the popular Caco-2 line, contain a mutated *p53* gene. Although all cell lines by definition have altered rates of proliferation and death, the IEC-6 line has proven useful to elucidate the pathways regulating apoptosis in untransformed crypt cells.

4. Polyamines and Apoptosis in Intestinal Cells

4.1. Polyamine Depletion Inhibits Apoptosis

Ray et al. *(21)* showed for the first time that polyamine depletion activates signal transduction pathways leading to cell-cycle arrest. Incubating IEC-6 cells with DFMO for 4 d inhibited proliferation, and arrested cells in the G_1 phase of the cell cycle. Cell-cycle arrest was accompanied by an increase in the level of p53 protein and the cell-cycle inhibitors, $p21^{Waf1/Cip1}$ and $p27^{Kip1}$. Unlike findings in many other cell lines, the induction of p53 did not induce apoptosis. p53 is activated by a variety of stimuli in

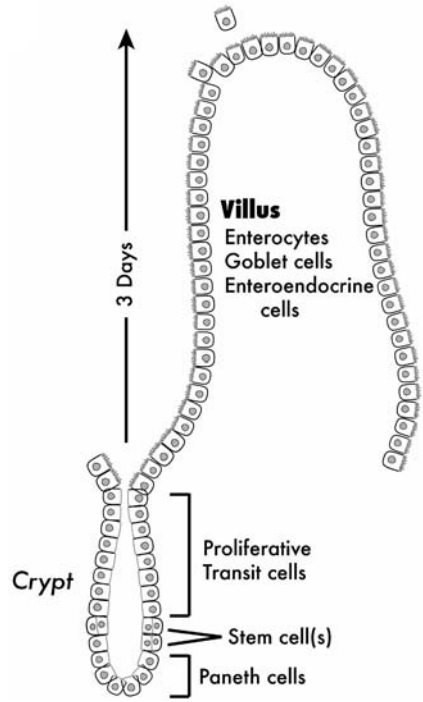

Fig. 3. The crypt-villus unit of the small intestine.

different cell types. These stimuli include DNA damage, withdrawal of growth factors, and the expression of Myc *(22)*. Whether apoptosis occurs depends on the severity of damage and other existing factors. The mechanisms involved in determining whether cell-cycle arrest or apoptosis occurs aren't entirely known, but the deletion of $p21^{Waf1/Cip1}$ can cause cells to undergo apoptosis that would otherwise have entered into cell-cycle arrest and repair *(22)*. Because damage-induced apoptosis of intestinal cells is p53-dependent *(23)*, we predicted that polyamine-deficient IEC-6 cells might be protected from apoptosis.

We examined the involvement of polyamines in the induction of apoptosis by the DNA topoisomerase I inhibitor, camptothecin *(24)*. In IEC-6 cells, camptothecin-induced apoptosis occurred within 6 h, accompanied by detachment of cells and an increase in caspase 3 activity. DFMO decreased the number of floating cells by 80% and inhibited the increase in caspase 3 activity by 75%. The addition of 10 μ*M* putrescine to the incubate along with DFMO prevented all the effects of polyamine depletion. Similar results were obtained when apoptosis was stimulated by TNF-α and cycloheximide (CHX) *(24)*. The inhibition of *S*-adenosylmethione decarboxylase with 1 m*M* diethylglyoxal *bis*-(guanyl-hydrozone) doubled the rate of apoptosis in response to camptothecin, while significantly increasing cell putrescine levels and depleting spermidine and spermine. Thus in this system, putrescine itself appears sufficient to

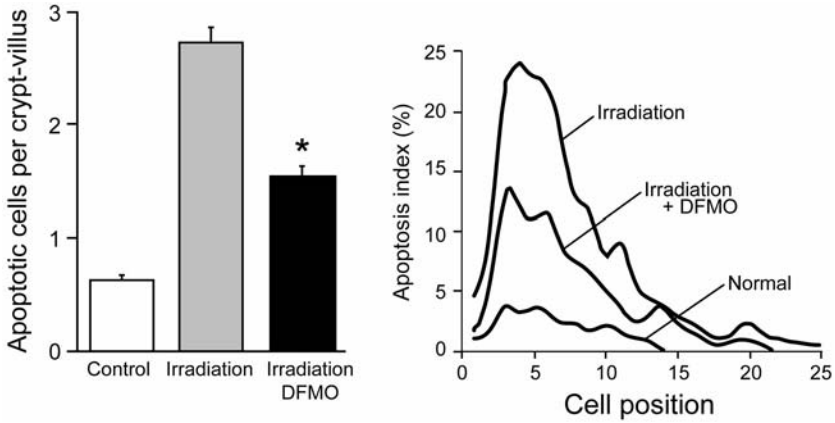

Fig. 4. Apoptotic cells in mice subjected to 15 Gy γ-irradiation and killed 4 h later. Mice received vehicle (control) or 2% DFMO in their drinking water for 4 d before irradiation. **(A)** Apoptotic cells per crypt-villus unit. **(B)** Position of apoptotic cells along the crypt villus axis. (Reproduced with permission from ref. *25*.)

support normal levels of apoptosis. This study also demonstrated that polyamine depletion was able to inhibit apoptosis, regardless of whether it was initiated by the intrinsic (camptothecin) or the extrinsic (TNF-α) pathway.

Polyamine depletion has the same effect on apoptosis in vivo as it does in vitro *(25)*. Pretreatment of IEC-6 cells with DFMO for 4 d significantly reduced radiation-induced apoptosis, as determined by DNA fragmentation and caspase 3 activity. Radiation exposure in mice resulted in a high frequency of apoptosis over cell positions 4 through 7 in the crypt (Fig. 4A). Pretreatment of mice with 2% DFMO in drinking water significantly reduced apoptotic cells from approx 2.75 to 1.61 per crypt-villus unit (Fig. 4B), accompanied by significant decreases in caspase 3 levels. Pretreatment with DFMO also inhibited the radiation-induced increase in the proapoptotic protein, Bax. Moreover, DFMO significantly enhanced the intestinal crypt survival rate by 2.1 times as determined 4 d after irradiation *(25)*. Thus these experiments indicate that the IEC-6 cell line reflects the responses to apoptosis and polyamines that occur in the intestine of the whole animal.

4.2. Parameters of Apoptotic Pathways Altered by Polyamine Depletion

Figure 5 indicates the apoptotic pathways delineated in IEC-6 cells and the references to the effects of polyamines on the specific components of those pathways. Yuan et al. *(26)* found that polyamine depletion prevented the release of cytochrome c from mitochondria after camptothecin. This was accompanied by increased Bcl-2 and Bcl-X_L and decreased Bax and *t*-Bid in the cells incubated with DFMO. In addition to inhibiting caspase 3 activation, DFMO significantly reduces the activation of caspase 6, 8, and 9 in response to TNF-α/CHX *(27)*. Thus, polyamine depletion inhibits apoptosis by decreasing both the extrinsic and intrinsic pathways. The remaining proapoptotic step shown in Fig. 5 involves JNK, which is activated by TNF-α through the TNFR1

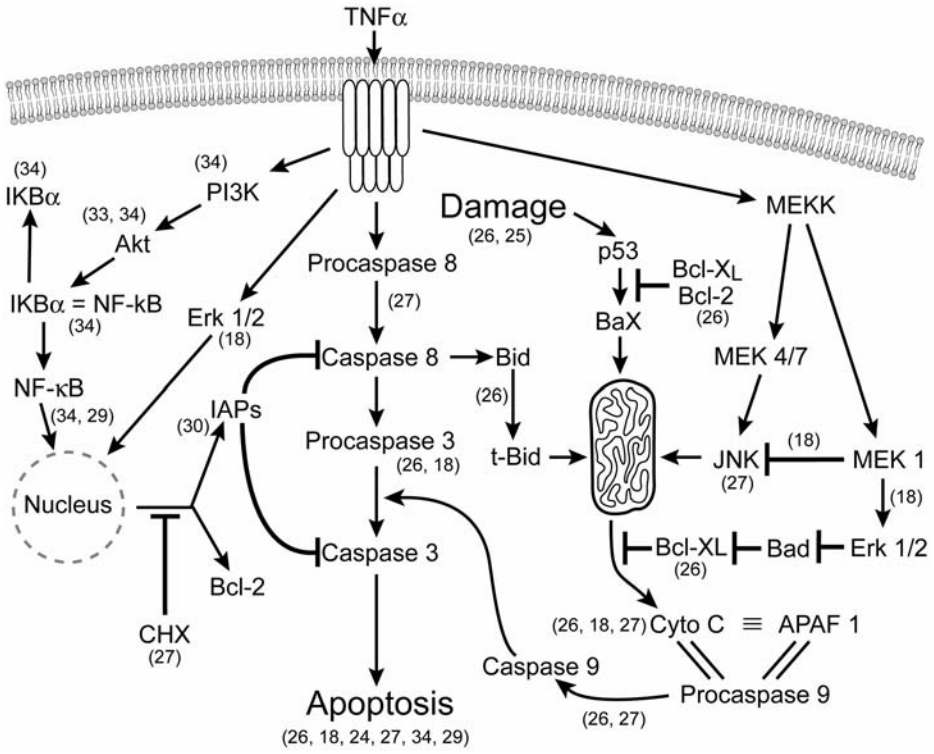

Fig. 5. Apoptotic pathways in IEC-6 cells activated by TNF-α/CHX and by damage induced by camptothecin or γ-irradiation. Reference numbers refer to articles demonstrating changes in a particular pathway component and the effect of polyamine depletion on it.

receptor and TRAF2. TNF-α also activates JNK in IEC-6 cells, and that activation is dependent on polyamines *(27)*. Polyamine depletion inhibits JNK activation, and inhibition of JNK with the specific inhibitor, SP-600125, prevented apoptosis, caspase 9 activation, and cytochrome *c* release. The mechanism by which JNK activates apoptosis is not clear; however, the results indicate that JNK acts upstream from cytochrome c release, possibly through the Bcl-2 family proteins.

Polyamine depletion causes the sustained activation of ERK in response to TNF-α/CHX, and the ERKs mediate a strong antiapoptotic response *(18)*. Pretreatment of polyamine-depleted cells with the membrane permeable MEK 1/2 inhibitor, U-0126, inhibited TNF-α-induced phosphorylation of ERK and increased DNA fragmentation, JNK activity, and caspase 3 activity. IEC-6 cells expressing constitutively active MEK1 were protected from apoptosis and had decreased JNK activity in response to TNF-α. Conversely, dominant-negative MEK1 cells had high basal levels of JNK activity, cytochrome c release, and spontaneous apoptosis. Depletion of polyamines in dominant-negative MEK1 cells did not prevent JNK activation, cytochrome c release, or apoptosis in response to TNF-α/CHX on camptothecin *(18)*.

Polyamine depletion rapidly activates NF-κB *(28)*. Within 1 h of administering DFMO, p65 rapidly redistributes from the cytoplasm to the nucleus of IEC-6 cells, and the degradation of IkBα is complete by 5 h. These times correlated with a 50% drop in putrescine levels at 1 h and a total absence of putrescine by 3 h. All of these early changes were prevented by the addition of putrescine to the cells incubated with DFMO *(28)*. Li et al. *(29)* also reported that polyamine depletion led to the activation of NF-κB from 4 to 8 d after constant exposure to DFMO. They showed that inhibition of NF-κB-binding activity by MG-132 or sulfasalazine prevented the increased apoptosis to staurosporine in polyamine-depleted cells and the inhibition of apoptosis in the same cells in response to TNF-α/CHX. Wang and his coworkers followed this report by showing that activation of NF-κB by polyamine depletion led to the synthesis of c-IAP2 and XIAP, as well as the inhibition of caspase 3 activity *(30)*. They did not attempt to reconcile these findings with their data indicating that NF-κB activation enhanced staurosporine-induced apoptosis *(29)*. This observation that DFMO increased staurosporine-induced apoptosis in IEC-6 cells remains the only report of enhanced apoptosis in IECs following depletion of polyamines. The mechanism leading to this effect is also unknown.

TNF-α-induced activation of NF-κB requires Akt, a serine/threonine protein kinase originally identified as the oncogene transduced by the acute transforming retrovirus Akt *(31)*. After cytokine stimulation, Akt translocates to the inner surface of the plasma membrane, where it is phosphorylated and activated by PI3K (Fig. 5). Although Akt inhibits apoptosis by preventing the release of cytochrome c, the mechanism is not clear. Glycogen synthase kinase-3β (GSK-3β), an important downstream target of Akt, is inhibited by Akt phosphorylation. Increased activity of GSK-3β has been shown to induce apoptosis in several cell types *(32)*. Zhang et al. *(33)* found that inhibition of Akt increases apoptosis in polyamine depleted IEC-6 cells, and suggested that Akt suppresses caspase 3 activity through a process involving the phosphorylation of GSK-3β. Bhattacharya et al. *(34)* found that the inhibition of GSK-3β had no effect on apoptosis induced by TNF-α in IEC-6 cells. They went on to show that inhibition of PI3-kinase and overexpressing of DN-Akt significantly increased apoptosis in polyamine-depleted cells. Constitutive activation of Akt by polyamine depletion was prevented in DN-Akt cells, thereby reversing the protective effect of polyamine depletion. Inhibitors of PI3 kinase and expression of DN-Akt prevented Akt activation and the subsequent translocation of NF-κB to the nucleus *(34)*. CA-Akt expression increased the resistance to TNF-α-induced apoptosis and increased the activation of NF-κB. Polyamine depletion of cells transfected with DN-Akt prevented spontaneous and TNF-α-induced IkBα phosphorylation. Prevention of NF-κB activation in cells transfected with DN-IkBα increased spontaneous apoptosis and restored it in polyamine-depleted cells. Thus protection from apoptosis by Akt in polyamine-deficient cells is dependent on the activation of the transcription factor, NF-κB, and is independent of GSK-3β. Akt regulates the mitochondrial pathway, preventing the activation of caspase 9 and, hence, caspase 3 via the actions of NF-κB *(34)*.

Signal transducers and activators of transcription (STAT) proteins are a family of latent transcription factors that undergo ligand-dependent phosphorylation and activation.

STAT3 activates the acute phase response genes and binds to the SIE (sis-inducible element) found in the *c-fos* promoter that is regulated by PDGF platelet-derived growth factor (PDGF). Pfeffer et al. *(35)* found that DFMO rapidly induced STAT3 activation as determined by its tyrosine phosphorylation, translocation from the cytoplasm to the nucleus, and presence in SIE-dependent DNA–protein complexes. Activation of STAT3 resulted in the activation of a STAT3-dependent reporter construct. Additional phosphorylation of STAT3 at serine 727 is thought to increase its transcriptional activity and is mediated by the mitogen-activated protein kinases pathway. STAT3 is constitutively activated in a wide variety of primary tumors and induces cell survival in association with survivin expression in gastric cancer cells *(36)*. STAT3 signaling has been reported to mediate the survival of intestine epithelial cells transfected with oncogenic Ras. Thus, the role of STAT3 as a mediator of cell survival is well established.

Bhattacharya et al. *(37)* determined that polyamine depletion induced STAT3 phosphorylation at both Tyr-705 and Ser-727, resulting in localization to the cell periphery and nucleus, respectively. Sustained phosphorylation of STAT3 at both residues occurred after polyamine-depleted cells were exposed to TNF-α. Inhibition of STAT3 increased the sensitivity of polyamine-depleted cells to apoptosis, and the protective effect of polyamine depletion was eliminated in cells expressing DN-STAT3. Additional evidence was obtained, indicating that DFMO increased both the transcription and translation of antiapoptotic proteins, Bcl-2, Mcl-1, and BIRC3. The levels of these proteins were significantly reduced in cells expressing DN-STAT3. Therefore, the activation of STAT3 and its subsequent effects as a transcription factor appear to be an important part of the mechanism that protects polyamine-depleted cells from apoptosis. This protection may be mediated by the expression of antiapoptotic proteins Bcl-2, Mcl-1, and BIRC3 *(37)*.

4.3. Regulation of Apoptotic Pathways in Polyamine-Depleted Cells

It would be naïve to believe that polyamine depletion directly alters each of the steps or even several of the steps depicted in Fig. 5. Numerous cellular processes, including apoptosis, are regulated by reversible protein phosphorylation by protein kinases and phosphatases in eukaryotic cells. The kinases themselves are dephosphorylated and inactivated by a type 2A protein phosphatase. Previous studies have implicated serine/threonine protein phosphatase-2A (PP2A) in a wide variety of cellular functions, including metabolism, proliferation, differentiation, ion transport, transcription, translation, and apoptosis *(38)*.

Figure 5 contains two major pathways, the ERK-JNK pathway and the PI3K-Akt-NF-κB pathway, which are altered during polyamine depletion. In addition, each of these cascades controls the levels or activities of Bcl-2 family proteins that are responsible for determining whether cytochrome c is released from mitochondria. The activities of many of the components of these pathways, and the Bcl-2 proteins themselves, are regulated by serine/threonine phosphorylation. Bcl-2, for example, is inactivated by dephosphorylation via PP2A, whereas Bad is released from its inactive state and bound to protein 14-3-3 by dephosphorylation, so it is activated by PP2A. A compilation of the effects of serine/threonine phosphorylation on the activities of key proteins in the

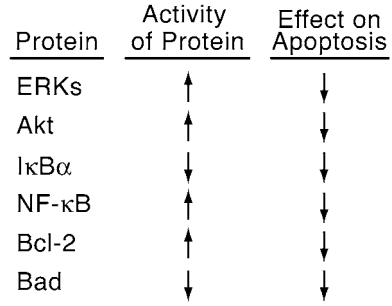

Fig. 6. The effects of serine/threonine phosphorylation on the activities of proteins involved in the apoptotic cascade. These effects are produced by inhibiting PP2A and by polyamine depletion. In each case the change in activity acts to decrease apoptosis.

apoptotic signaling pathways is shown in Fig. 6. In each case, the effect is in the same direction as that caused by the depletion of polyamines. And, in each case, the change in activity, whether an increase or decrease, acts to prevent apoptosis.

TNF-α activates the Akt and ERK survival pathways and apoptotic pathways (Fig. 5). In control cells and those exposed to putrescine plus DFMO, these activities peak around 3–4 h and then decrease to basal levels by 6–9 h *(18,34)*. In polyamine-depleted cells, however, the activities of these enzymes increase from their already elevated levels to higher levels, and then plateau. Thus there appears to be no inactivation of Akt and ERK in DFMO-treated cells, so that polyamine depletion renders these pathways constitutively active. These observations plus those shown in Fig. 6 could be explained if PP2A were inhibited in polyamine-depleted cells and if its inactivation protected cells from apoptosis. There is some support for this hypothesis because earlier reports indicate that polyamines can activate PP2A specifically compared with PP1 *(39,40)*.

In examining this explanation, Ray et al. *(41)* found that polyamine-depleted cells had only one-third the PP2A activity of control cells or those incubated with putrescine in addition to DFMO. Inhibition of PP2A with either okadaic acid or fostriecin protected IEC-6 cells from apoptosis induced by TNF-α/CHX. Cells transfected with the small interfering RNA for PP2A were also protected. Inactivation of Bad involves the phosphorylation of serine at residues 112 and 136, which decreases its affinity for Bcl-X_L at the mitochondrial membrane, freeing Bcl-X_L to prevent apoptosis. PP1 activates Bad by serine dephosphorylation. Current data indicate that PP1 itself is dephosphorylated and activated by PP2A, which is also responsible for dephosphorylating and inactivating Bcl-2 *(38)*. Thus decreased PP2A activity in polyamine-depleted cells may not only prevent the inactivation of Bcl-2, but may also cause Bad to remain phosphorylated and inactive. We found significantly higher levels of pSer112-Bad in polyamine-depleted cells compared with those grown under control conditions *(41)*. Furthermore, the level of active pSerBcl-2 decreased after exposure of control cells to TNF-α/CHX, but increased significantly in those incubated with DFMO. In the same study, okadaic acid increased the phosphorylation of both these proteins similar to the effects of polyamine depletion. Inhibiting PP2A with okadaic acid also mimicked other

effects of polyamine depletion, such as the prevention of cytochrome c release, inhibition of JNK activity, and the inhibition of caspases 9 and 3 in response to TNF-α *(41)*. PP2A, therefore, is a crucial point for controlling apoptosis in IECs.

PP2A itself is regulated by tyrosine kinases, such as Src-kinase and the EGF receptor, which phosphorylate it, inhibiting the enzyme *(42)*. Signals transduced through a single receptor, such as the TNFR, can lead either to cell proliferation or apoptosis depending on how the cell is programmed to respond. Src activation is antiapoptotic, and expression of v-Src rescues several cell types from apoptosis induced by a variety of stimuli, including cytokine withdrawal, irradiation, chemotherapeutic drugs, and detachment from the extracellular matrix *(43,44)*. These observations suggest that constitutively activated Src can mimic the effects of cytokine receptors, integrins, and other receptor pathways that protect cells from apoptosis. The mechanism by which Src inhibits apoptosis is not well defined because of the divergent nature of Src signaling. However, current studies suggest that Src may regulate apoptosis by altering the strength and balance of signals and controlling the various mitogen-activated protein kinases and the PI3 kinase-Akt pathways *(45,46)*.

We have recently found that Src is constitutively active in polyamine-depleted cells *(47)*. Phosphorylation of Tyr 416 in the kinase domain completely activates Src and provides a binding site for SH_2 domains of other proteins *(48)*. Incubation of IEC-6 cells with DFMO for 4 d significantly increased the amount of pY416-Src without altering the amount of Src protein. Pharmacological inhibition of Src increased the rate of spontaneous apoptosis and that induced by TNF-α in control cells. In polyamine-depleted cells, inhibiting Src decreased Akt and STAT3 activities, blocked the increased activation of Bcl-2 and Bcl-X_L, and restored apoptosis to control levels. The expression of DN-Src increased apoptosis significantly and eliminated protection conferred by polyamine depletion. On the other hand, the expression of CA-Src produced cells resistant to apoptosis *(47)*. Figure 7 illustrates a model depicting how the activation of Src might account for the resistance to apoptosis in polyamine-depleted cells.

As discussed previously, the activation of STAT3 contributes to the resistance of polyamine-depleted cells to apoptosis. Interestingly, Src activation may also be involved in the activation of STAT3. Src has been shown to associate with STAT3 and be responsible for its activation in various transformed cells *(49,50)*. Zhang et al. *(51)* have found evidence for and proposed a mechanism in which Src, as part of a membrane anchored scaffold, phosphorylates and activates JAK, which in turn phosphorylates the cytoplasmic tail of a membrane receptor, which acts as the docking site for STAT3, which is then phosphorylated and activated by Src. The activation of STAT3 by Src is also shown in Fig. 7.

5. Conclusions

If the scheme hypothesized in Fig. 7 proves correct, the obvious unanswered question are: how does the removal of polyamines result in Src activation? Could polyamines prevent the translocation of Src to the membrane? Could they block its activation site? Obviously, there are as many possibilities as there are steps in the activation process. The answers to these questions could prove interesting and important to our understanding not only of polyamine interactions, but also apoptosis itself.

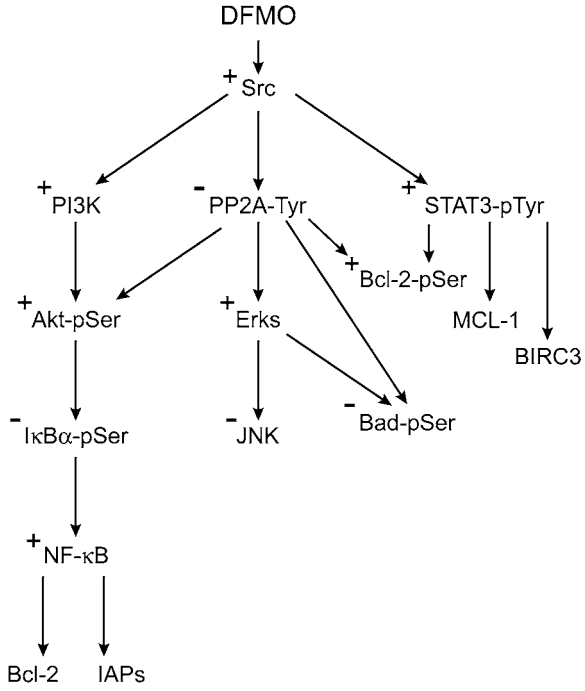

Fig. 7. The effects of polyamine depletion (DFMO) on pathways regulating apoptosis. Activation of Src leads to the activation of transcription factors NF-κB, ERK, and STAT3. Src phosphorylates PP2A, inactivating it, which prevents it from inactivating Akt, ERK, and Bcl-2. In addition, PP2A is unable to dephosphorylate IkBα and Bad, keeping them in the inactive state. Plus and minus signs refer to the activity of the individual molecules.

That polyamine depletion decreases apoptosis in vivo and in vitro suggests the possibility that DFMO may have an as-yet untested use in cancer therapy. For some time, DFMO has been used as an adjunct to chemotherapy to kill cancer cells—with mixed results. One of the major limiting factors in cancer therapy is the so-called gastrointestinal syndrome caused by the therapeutic agent inducing apoptosis in the rapidly growing normal enterocytes and the cancer cells. These patients develop nausea, diarrhea, and lose weight, limiting the doses, duration, and effectiveness of their therapy. It may be possible to provide some degree of protection by administering DFMO orally to deplete these cells of polyamines before the exposure to therapeutic chemicals.

References

1. Jäne, J., Alhonen, L., Pietilä, M., and Keinänen, T. A. (2004) Genetic approaches to the cellular functions of polyamines in mammals. *Eur. J. Biochem.* **271,** 877–894.
2. Schipper, R. G., Penning, L. C., and Verhofstad, A. A. J. (2000) Involvement of polyamines in apoptosis. Facts and controversies: effectors or protectors? *Semin. Cancer Biol.* **10,** 55–68.

3. Quaroni, A., Wands, J., Trelstad, R. L., and Isselbacher, K. J. (1979) Epithelioid cell cultures from rat small intestine. *J. Cell Biol.* **80,** 248–265.
4. McCormack, S. A., Viar, M. J., and Johnson, L. R. (1993) Polyamines are necessary for cell migration by a small intestinal crypt cell line. *Am. J. Physiol. Gastrointest. Liver Physiol.* **264,** G367–G374.
5. Ziegler, U. and Groscurth, P. (2004) Morphological features of cell death. *News Physiol. Sci.* **19,** 124–128.
6. Thornberry, N. A. and Lazebnik, Y. (1998) Caspases: enemies within. *Science* **281,** 1312–1316.
7. Liu, X., Li, P., Widlak, P., et al. (1998) The 40 kDa subunit of DNA fragmentation factors induces DNA fragmentation and condensation during apoptosis. *Proc. Natl. Acad. Sci. USA* **95,** 8461–8466.
8. Nicholson, D. W. and Thornberry, N. A. (2003) Life and death decisions. *Science* **299,** 214–215.
9. Deveraux, Q. L., Leo, E., Steinnicke, H. R., Welsh, K., Salvesen, G. S., and Reed, J. C. (1999) Cleavage of human inhibitors of apoptosis protein XIAP results in fragments with distinct specificities for caspases. *EMBO J.* **18,** 5242–5251.
10. Wu, G., Chai, J., Suber, T. L., et al. (2000) Structural basis of IAP recognition of Smac/Diablo. *Nature* **408,** 1008–1012.
11. Kelekar, A. and Thompson, C. B. (1998) Bcl-2 family proteins: the role of the BH_3 domain in apoptosis. *Trends Cell. Biol.* **8,** 324–330.
12. Oltvai, Z. N., Milliman, C. L., and Korsmeyer, S. J. (1993) Bcl-2 heterodimerizes with a conserved homolog, Bax, that accelerates programmed cell death. *Cell in Vivo* **74,** 609–619.
13. Ledgerwood, E. C., Pober, J. S., and Bradley, J. R. (1999) Recent advances in the molecular basis of TNF signal transduction. *Lab. Invest.* **79,** 1041–1050.
14. Hsu, H., Shu, H. -B., Pan, M. -G., and Goedeel, D. V. (1996) TRADD-TRF2 and TRADD-FADD interaction define two distinct TNF receptor 1 signal transduction pathways. *Cell* **84,** 299–309.
15. Beg, A. A. and Baltimore, D. (1996) An essential role for NF-κB in preventing TNF-α induced cell death. *Science* **274,** 782–784.
16. Lewis, T. S., Shapiro, P. S., and Ahn, N. G. (1998) Signal transduction through MAPK cascades. *Adv. Cancer Res.* **74,** 49–139.
17. Reinhard, C., Shamoon, B., Shyamala, V., and Williams, L. T. (1997) TNFα-induced activation of JNK is mediated by TRAF2. *EMBO J.* **16,** 1080–1092.
18. Bhattacharya, S., Ray, R. M., and Johnson, L. R. (2004) Prevention of TNF-α-induced apoptosis in polyamine-depleted IEC-6 cells is mediated through the activation of ERK 1/2. *Am. J. Physiol. Gastrointest. Liver Physiol.* **286,** G479–G490.
19. Wright, N. A. and Irwin, M. (1982) The kinetics of villus cell populations in the mouse small intestine: normal villi-the steady state requirement. *Cell Tissue Kinet.* **15,** 595–609.
20. Grossman, J., Walther, K., Artinger, M., Kiessling, S., and Scholmerich, J. (2001) Apoptotic signaling during initiation of detachment induced apoptosis ("anoikis") of primary human intestinal epithelial cells. *Cell Growth Differ.* **12,** 147–155.
21. Ray, R. M., Zimmerman, B. J., McCormack, S. A., Patel, T. B., and Johnson, L. R. (1999) Polyamine depletion arrests cell cycle and induces inhibitors $p21^{waf1/cip1}$, $p27^{kip1}$ and p53 in IEC-6 cells. *Am. J. Physiol. Cell Physiol.* **276,** C684–C691.
22. Agarwal, M. L., Taylor, W. R., Chernov, M. V., Chernova, O. B., and Stark, G. R. (1998) The p53 network. *J. Biol. Chem.* **273,** 1–4.
23. Merritt, A. J., Potten, C. S., Kemp, C. J., et al. (1994) The role of p53 in spontaneous and radiation-induced apoptosis in the gastrointestinal tract of normal and p53-deficient mice. *Cancer Res.* **54,** 614–617.

24. Ray, R. M., Viar, M. J., Yuan, Q., and Johnson, L. R. (2000) Polyamine depletion delays apoptosis of rat intestinal epithelial cells. *Am. J. Physiol. Cell Physiol.* **278,** C480–C489.
25. Deng, W. and Johnson, L. R. (2005) Polyamine depletion inhibits irradiation-induced apoptosis in intestinal epithelia. *Am. J. Physiol. Gastrointest. Liver Physiol*. **289,** G599–G606.
26. Yuan, Q., Ray, R. M., and Johnson, L. R. (2002) Polyamine depletion prevents camptothecin-induced apoptosis by inhibiting the release of cytochrome *c*. *Am. J. Physiol. Cell Physiol.* **282,** C1290–C1297.
27. Bhattacharya, S., Ray, R. M., Viar, M. J., and Johnson, L. R. (2003) Polyamines are required for the activation of *c-Jun* NH_2-terminal kinase and apoptosis in response to TNF-α in IEC-6 cells. *Am. J. Physiol. Gstrointest. Liver Physiol.* **285,** G980–G991.
28. Pfeffer, L. M., Yang, C. H., Murti, A., et al. (2001) Polyamine depletion induces rapid NF-κB activation in IEC-6 cells. *J. Biol. Chem.* **276,** 45,909–45,913.
29. Li, L., Rao, J. N., Bass, B. L., and Wang, J.-Y. (2001) NF-κB activation and susceptibility to apoptosis after polyamine depletion in intestinal epithelial cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* **280,** G992–G1004.
30. Zou, T., Rao, J. N., Guo, X., et al. (2004) NF-(kappa)B-mediated IAP expression induces resistance of intestinal epithelial cells to apoptosis after polyamine depletion. *Am. J. Physiol. Cell Physiol.* **286,** C1009–C1018.
31. Madrid, L. V., Wang, C. Y., Guttridge, D. C., Schottelins, A. J., Baldwin, A. S. Jr., and Mayo, M. W. (2000) Akt suppresses apoptosis by stimulating the transactivation potential of the Rel/p65 subunit of NF-kappa B. *Mol. Cell Biol.* **20,** 1626–1638.
32. Pap, M. and Cooper, G. M. (1998) Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-kinase/Akt survival pathway. *J. Biol. Chem.* **273,** 19,929–19,932.
33. Zhang, H. M., Rao, J. N., Guo, X., et al. (2004) Akt kinase activation blocks apoptosis in intestinal epithelial cells by inhibiting caspase-3 after polyamine depletion. *J. Biol. Chem.* **279,** 22,539–22,547.
34. Bhattacharya, S., Ray, R. M., and Johnson, L. R. (2005) Decreased apoptosis in IEC-6 cells following polyamine depletion depends on NF-κB activation but not GSK3β activity. *Apoptosis* **10,** 759–776.
35. Pfeffer, L. M., Yang, C. H., Pfeffer, S. R., Murti, A., McCormack, S.,A., and Johnson, L. R. (2000) Inhibition of ornithine decarboxylase induces STAT3 tyrosine phosphorylation and DNA binding in IEC-6 cells. *Am. J. Physiol. Cell Physiol.* **278,** C331–C335.
36. Kanda, N., Seno, H., Konda, Y., and Chiba, T. (2004) STAT3 is constitutively activated and supports cell survival in association with surviving expression and in gastric cancer cells. *Oncogene* **23,** 4921–4929.
37. Bhattacharya, S., Ray, R. M., and Johnson, L. R. (2005) STAT3-mediated transcription of Bcl-2, Mcl-1 and BIRC3 prevents apoptosis in polyamine-depleted cells. *Biochem. J*. **392,** 335–344.
38. Garcia, A., Cayla, X., Guergnon, J., et al. (2003) Serine/threonine protein phosphatases PP1 and PP2A are key players in apoptosis. *Biochimie* **85,** 721–726.
39. Tung, H. Y., Pelech, S., Fisher, M. J., Pogson, C. I., and Cohen, P. (1985) The protein phosphatases involved in cellular regulation. Influence of polyamines on the activities of PP-1 and PP2A. *Eur. J. Biochem.* **149,** 305–313.
40. Cornwell, T., Mehta, P., and Shenolikar, S. (1986) Polyamine stimulation of PP-2A from rat liver using a non-protein phosphoester substrate. *J. Cyclic Nacleotide Protein Phosphor. Res.* **11,** 373–382.
41. Ray, R. M., Bhattacharya, S., and Johnson, L. R. (2005) Inhibition of PP2A in polyamine depleted cells is responsible for their resistance to apoptosis. *J. Biol. Chem*. **280,** 31,091–31,100.

42. Chen, J., Martin, B. L., Brautigan, D. L. (1992) Regulation of protein serine-threonine phosphatase type 2A by tyrosine phosphorylation. *Science* **257,** 1261–1264.
43. Pengetnze, Y., Steed, M., Roby, K. F., Terranova, P. F., and Taylor, C. C. (2003) Src tyrosine kinase promotes survival and resistance to chemotherapeutics in a mouse ovarian cancer cell line. *Biochem. Biophys. Res. Commun.* **309,** 377–383.
44. Anderson, S. M., Carroll, P. M., and Lee, F. D. (1990) Aborgation of IL-3 dependent growth requires a functional v-Src gene product. *Oncogene* **5,** 317–325.
45. Gardner, A. M., and Johnson, G. L. (1996) Fibroblast growth factor-2 suppression of TNFα-mediated apoptosis requires Ras and the activation MAP kinase. *J. Biol. Chem.* **271,** 14,560–14,566.
46. Yao, R. and Cooper, R. M. (1995) Requirement for PI-3 kinase in the prevention of apoptosis by NGF. *Science* **267,** 2003–2006.
47. Bhattacharya, S., Ray, R. M., and Johnson, L. R. Activation of Src regulates apoptosis in intestinal epithelial cells. In press.
48. Brown, M. T., and Cooper, J. A. (1996) Regulation, substrates, and functions of Src. *Biochim. Biophys. Acta* **1287,** 121–149.
49. Gracia, R., Bowman, T. L., Guillian, N., et al. (2001) Constitutive activation of STAT3 by the Src and JAK tyrosine kinases participates in growth regulation of human breast carcinoma cells. *Oncogene* **20,** 2499–2513.
50. Cao, X., Tay, A., Guy, G. R., and Tan, Y. H. (1996) Activation and association of STAT3 with Src in v-Src transformed cell lines. *Mol. Cell Biol.* **16,** 1595–1603.
51. Zhang, Y., Turkson, J., Carter-Su, C., et al. (2000) Activation of STAT3 in v-Src-transformed fibroblasts requires cooperation of JAK1 kinase activity. *J. Biol. Chem.* **275,** 24,935–24,944.

14

The Role of Ornithine Decarboxylase in Myc-Induced Tumorigenesis

Jonas A. Nilsson and John L. Cleveland

1. Introduction

Myc oncogenes (c-*Myc* and its relatives N-*Myc* and L-*Myc*) are activated in a large cast of human cancers, as much as 70% of all malignancies. Thus, understanding how Myc contributes to tumor formation is a major focus of oncology research. The first *Myc* gene discovered was a viral oncogene, v-*myc*, the cancer-causing gene of the MC29 avian myelocytomatosis virus *(1)*. Using v-*myc* as a probe, the cellular homolog *MYC* (c-*Myc* in mouse) was then identified *(2,3)*, which turned out to be the very same gene translocated and juxtaposed to the immunoglobulin enhancers in human Burkitt lymphoma and in mouse plasmacytoma *(4,5)*. Myc oncoproteins were subsequently found to be key regulators of the cell cycle *(6–8)* and the creation of their knockouts in mice revealed that c-*Myc* and N-*Myc* (but not L-*Myc*) are essential for mammalian development because c-Myc- and N-Myc-deficient embryos fail at mouse embryonic days E9.5 and E11.5, respectively *(9–15)*. Analyses of these mice, and other cell-based studies, also established central roles for these oncoproteins as regulators of cell growth (i.e., cell mass) differentiation, cell adhesion, and vasculogenesis and angiogenesis *(15–21)* (Fig. 1).

Myc genes respond to extracellular mitogens as classical primary response genes and, conversely, *Myc* transcription is suppressed by signals that inhibit the growth of cells. *Myc* gene transcription is regulated by numerous signaling pathways known to be altered in cancer, including those directed by epidermal growth factor receptors, transforming growth factor-β, nuclear factor-κB, and Wnt and β-catenin *(22,23)*. In addition, by mechanisms not fully understood, these signaling pathways, and others, can also stabilize *myc* transcripts, promote c-Myc translation, and alter the half-life of the protein *(24)*. Finally, specific posttranslational modifications, including both phosphorylation and glycosylation *(25–36)*, regulate the activity of these oncoproteins. Notably, virtually every level of control of these oncoproteins has been shown to be altered in cancer.

In cancer, Myc levels are elevated in response to amplification (e.g., *MYCN* and *MYCL* in neuroblastoma *[6,8,37]* and lung cancer *[7]*, respectively), gene translocation

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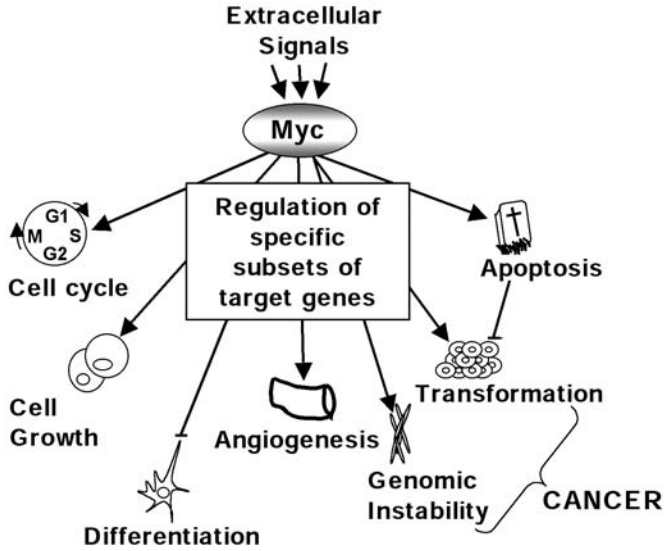

Fig. 1. Functions of Myc oncoproteins. Under physiological conditions, Myc contributes to the normal development of an organism by stimulating Myc target genes involved in cell proliferation, cell growth, cell division, and vasculogenesis and angiogenesis. When Myc is aberrantly expressed, as occurs in precancerous cells, apoptosis is triggered to protect cells from aberrant cell proliferation, that otherwise would lead to genomic instability that causes second hits that lead to overt malignancy (transformation).

(e.g., *MYC* in B-cell malignancies *[5]*), enhanced transcription because of deregulation of proteins that regulate *MYC* transcription (e.g., in colon cancer in response to loss of Adenomatous polyposis coli *[23]*), or by mutations that lead to enhanced stability or altered activity of the protein (e.g., somatic *MYC* mutations in Burkitt lymphoma *[26]*). In turn, elevated levels of Myc results in an uncontrolled cell proliferation and, in malignancies, to the tumor angiogenic response *(15,20,38,39)*. Given the vast array of events that can lead to Myc overexpression and that cancer is a rather rare incident in the life of an individual, it follows that cells possess intrinsic defense mechanisms that guard against elevated levels of Myc. Indeed, this checkpoint became evident when it was discovered that c-Myc *(40,41)* and other oncoproteins, such as E1A *(42–44)* and E2F-1 *(45–48)*, are potent inducers of cell suicide (apoptosis) when overexpressed in normal cells (Fig. 1). Myc-induced apoptosis is particularly germane to the tumor cell, as it is especially evident under circumstances when cells are deprived of essential growth factors, or are exposed to DNA damage or hypoxia. The mechanisms responsible for Myc-induced apoptosis have recently been reviewed in depth elsewhere *(49–51)*.

1.1. The Max Network

c-Myc, N-Myc, and L-Myc oncoproteins are members of a family of basic helix–loop–helix leucine zipper (bHLH-LZ) transcription factors that also includes

Myc's distant cousins B-Myc *(52)* and s-Myc *(53)*. All function within a network of bHLH-LZ factors, the "Max network" (Fig. 2), which includes the Myc proteins, the Mad proteins (Mad1, Mxi1, Mad3, and Mad4), Mntm, and Mga *(54–59)*. The central molecule in the network is the small bHLH-LZ factor Max, which interacts with other members through HLH-LZ interactions to form transcriptionally active heterodimers *(60,61)*. Max-containing dimers bind to E-box motifs (CAYGTG) present in the genome *(62–64)*, and the transcriptional outcome is dependent on the composition of the dimer. For example, Myc:Max dimers stimulate transcription through their associations with coactivators, such as TRRAP *(65–67)* and associated histone acetyltransferases, or with adenosine triphosphatase/helicase chromatin remodeling proteins *(65–69)*. By contrast, if Mad/Mnt:Max dimers predominate transcription is suppressed through the association Mad or Mnt with the transcriptional corepressors Sin3 and N-CoR, and their associated histone deacetylases (HDACs) *(55,70–73)* (Fig. 2).

To add yet further complexity, Myc:Max dimers can also inhibit the transcription of genes transcribed through initiator elements (Inr), as in the case of the transcription of the cyclin-dependent kinase (Cdk) inhibitors $p15^{INK4a}$ and $p21^{Cip1}$, and with platelet-derived growth factor. Inr elements are usually bound by the transcription factor Miz-1, which activates transcription via its associations with histone acetyltransferases. By binding to Miz-1, Myc interferes with this interaction, converting it to a transcriptional repression complex (Fig. 2) *(74–82)*, that can be further enhanced by the recruitment of DNA methyltransferase 3A *(83)*. These events are of particular importance in regard to $p15^{INK4a}$ and $p21^{Cip1}$, because they provide a mechanism by which Myc can override DNA damage ($p21^{Cip1}$) and transforming growth factor-β ($p15^{INK4a}$)-mediated cell-cycle arrest. A role of the Mad/Mnt family in Miz-1-mediated pathways has not yet been determined.

1.2. Myc Target Genes

Myc target genes can be generally classified in two ways: those that are repressed or induced by Myc or those that are direct vs indirect transcription targets. Identifying Myc targets has become a prodigious task because Myc regulates the expression of so many genes; indeed, a Web site has been established to keep track of all of these targets (http://www.myccancergene.org) *(84)*. Currently, 1697 Myc target genes have been identified using biased approaches and unbiased tools, such as Serial Analysis of Gene Expression and microarray *(85–94)*. Although already rather mind-blowing, recent experiments using chromatin immunoprecipitation and DamID assays actually indicate that the numbers of Myc targets is underestimated, and these studies are primarily focused on those targets having E-boxes in their promoter regulatory regions *(95–100)*. That most of these target genes have been identified in response to c-Myc overexpression also raises the question of whether they are only relevant in scenarios such as cancer, where Myc is overexpressed. Indeed, early attempts to identify genes that lose their normal expression pattern in response to serum in cells deficient in *c-Myc* only identified *cad*, out of the few established target genes tested, to be dependent on c-Myc for its expression *(101)*.

Germane to this review, Ornithine decarboxylase (*Odc*), S-adenosylmethionine decarboxylase (*Amd1*), and Spermidine synthase (*Srm*) are identified as targets in the

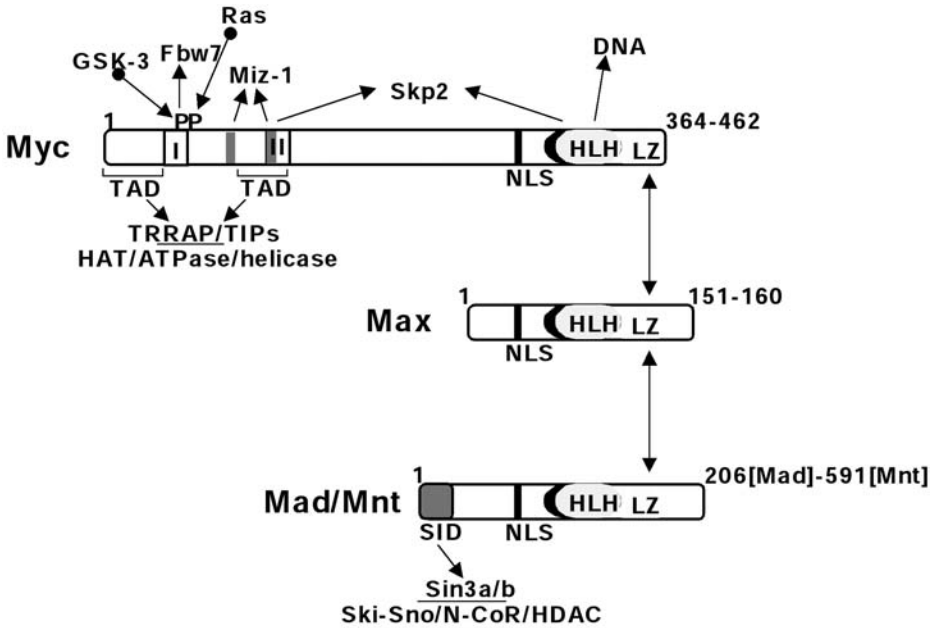

Fig. 2. The Max network. The functional domains of Max, and of the Myc and Mad families of proteins are shown. The N-termini of Myc and Mad family proteins direct their interactions with numerous proteins controlling protein stability and activity (for Myc: Ras, Fbw7, and Skp2) and transactivation/transrepression (for Myc: TRRAP and TIPs; for Mad: Sin3a and Sin3b). The C-termini of these transcription factors harbor the bHLH-LZ domain responsible for DNA binding (the basic domain, b) and the obligate dimerization domain (HLH-LZ) required for interactions with Max. The number of residues in these proteins is also indicated. HLH, helix–loop–helix; LZ, leucine zipper; NLS, nuclear localization signal; PP, phosphorylation sites; SID, Sin3-interacting domain; TAD, transactivation domain.

Myc target gene database. *Odc* was one of the first Myc target genes identified *(102–104)* and is the only gene encoding a polyamine biosynthetic enzyme that has been characterized in regard to Myc (despite the presence of several E-boxes in *Amd1* and *Srm*; Fig. 3). This chapter, therefore, focuses on the regulation and role of *Odc* in Myc-induced tumorigenesis, yet *Amd1* and *Srm* are certainly important candidates that deserve further scrutiny as mediators of Myc-induced pathways.

1.2.1. Myc Induces Odc

The mouse *Odc* gene contains 12 exons (Fig. 3) and is essential for embryonic development *(105)*. *Odc* is a direct target induced by Myc based on the following facts: (a) its promoter activity and transcript levels are elevated in cells overexpressing Myc *(102–104)*; (b) *Odc* transcripts are induced by Myc in the absence of *de novo* protein synthesis—this comes from analyses of cells expressing MycER, a chimeric form of Myc that can be activated by tamoxifen, in which Myc is fused to the ligand-binding

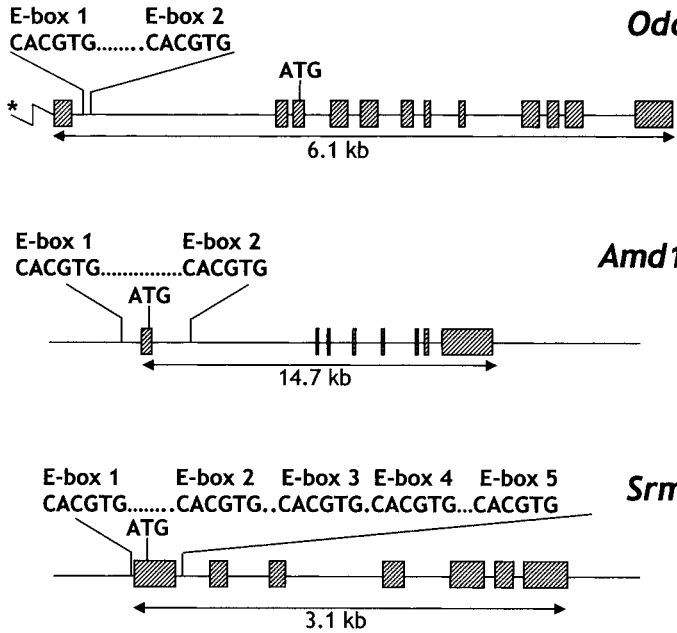

Fig. 3. Genomic structures of the genes encoding polyamine biosynthetic enzymes that are induced by Myc in B-cells of the Eμ-*Myc* mouse ***(123)***. Note the presence of canonical/classical E-boxes in all three genes.

domain of the estrogen receptor *(15,104)*; and (c) the *Odc* gene harbors two perfect (CACGTG), conserved, and functional E-boxes in intron 1 *(102,103)*, to which Myc binding can be detected by chromatin immunoprecipitation assays (ChIP assays) and in electromobility shift assays (*102,106)*. In addition, a third functional E-box was recently found upstream of the transcriptional start site of *Odc (107)*.

Our understanding of the mechanism by which Myc transactivates *Odc* (and likely other targets) has recently undergone some revisions *(108)*. Based on the fact that in quiescent or differentiating cells Myc levels are low and Mad levels are high, it was assumed that Mad family members antagonize Myc's ability to activate transcription *(54,70,71,109–112)*. However, when one adds Mnt into the equation this model changes. As with Mad factors, Mnt is a transcriptional repressor and can inhibit Myc-induced cell growth and transformation *(55,113)*. However, Mnt is coexpressed with Myc *(55)* and has even been identified as a Myc target in screens for Myc-responsive genes *(91)*. When analyzing the *Odc* promoter by ChIP assay and electromobility shift assay, we recently discovered that the E-boxes of *Odc* are not occupied by Mad or Myc but by Mnt *(106)*. Serum stimulation of quiescent fibroblasts resulted in a rapid appearance of Myc:Max complexes, which displaced Mnt:Max complexes from binding to the E-boxes of *Odc* intron 1, resulting in activation by relieving transrepression (Fig. 4). Interestingly, RNA interference-mediated knockdown of *Mnt (106)*, and the targeted deletion of *Mnt (114)*, recapitulated the biological effects of Myc overexpression in

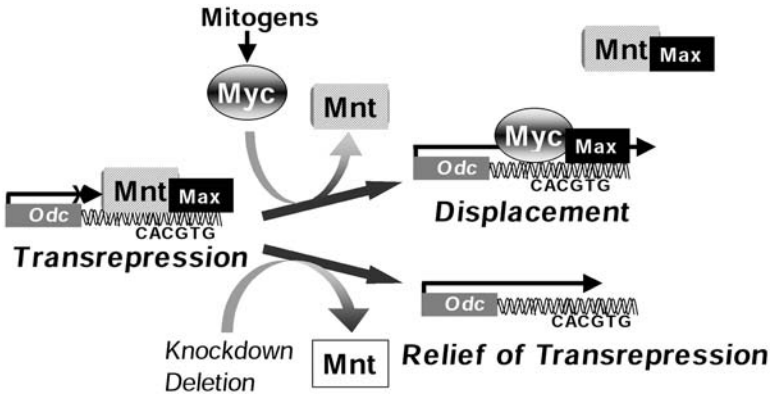

Fig. 4. Revised model of how Myc activates genes such as *Odc*. This model posits that Mnt:Max complexes bind the E-boxes of *Odc*. Mitogen stimulation leads to transient increases in Myc protein that then dimerizes with Max and displaces Mnt:Max complexes, resulting in induction of transcription by displacement or transactivation. However, removal of Mnt by knockdown or deletion is also sufficient to activate *Odc*, even in cells lacking *Myc*, suggesting that relief of transrepression is the mode of action, not classic transactivation *(106)*.

fibroblasts (Fig. 1); (i.e., *Mnt* loss enhanced cell proliferation and apoptosis, as well as transformation in conjunction with an activated Ras oncogene) *(106)*. Because these effects of *Mnt* RNA interference were also observed in *Myc*-null fibroblasts, this suggests that the function of Myc is to antagonize Mnt-mediated transrepression of target genes, such as *Odc* (Fig. 4). This model has been supported by very recent studies conditionally codeleting c-*Myc* and *Mnt* in mouse embryo fibroblasts, which again leads to the induction of many other Myc targets and to the restoration of cell growth *(115)*.

2. The Role of *Odc* in Myc-Induced Tumorigenesis

Studies evaluating the role of Odc in tumorigenesis have principally relied on the selective Odc enzyme inhibitor α-difluoromethylornithine (DFMO), which impairs tumor development in several mouse models of cancer, and appears to hold promise in the clinic *(116)*. However, in general these studies have been correlative, and they have not addressed the mechanism by which DFMO impairs tumor development. To determine the role of *Odc* in Myc-driven tumors we used the immunoglobulin heavy chain enhancer (Eμ)-*Myc* transgenic mouse, which overexpresses the c-*Myc* gene in B-cells by virtue of an Eμ. These mice, after a somewhat protracted precancerous phase, develop an oligoclonal, aggressive, and lethal B-cell lymphoma, and the mice usually succumb to their disease between 3 and 6 mo of age *(117)*. Although the transgenic construct faithfully recapitulates the t(8:14) translocation occurring in human Burkitt lymphoma *(5)*, the mice do not develop Burkitt lymphoma, but an aggressive pre-B/mature B-cell lymphoma *(117)*. Nevertheless, many of the secondary genetic hits that occur during tumorigenesis in this mouse model have now also been shown to occur in human Burkitt lymphoma *(118–121)*.

In the precancerous phase of the disease, the B-cells of Eμ-*Myc* mice exhibit accelerated rates of proliferation, yet this is kept in check by high rates of cell turnover through apoptosis *(117,122)*. Gene expression profiling established that a large number of Myc targets are turned on during this precancerous phase, and among these are *Odc*, *Amd1*, and *Srm*. In addition, *Sat1*, which encodes spermidine/spermine-*N*-acetyltransferase that directs polyamine catabolism is repressed in these cells *(123)*. The net result is an elevation in the levels of all polyamines in the B-cells of these mice. Importantly, by breeding the Eμ-*Myc* mice to mice lacking one allele of *Odc*, or by treating Eμ-*Myc* mice with DFMO, we established a critical role for Odc in Myc-induced lymphomagenesis because Eμ-*Myc*;*Odc*$^{+/-}$ mice and DFMO-treated animals had a greatly protracted course of disease and a threefold increase in their lifespan *(123)*. This is quite remarkable, given that *Odc* is just one of well over 1000 targets regulated by Myc. These findings thus underscore the pivotal roles polyamines play during Myc-driven tumorigenesis, and they also suggest that targeting other metabolic enzymes regulated by Myc may also be effective in cancer chemoprevention.

A curiosity coming from these studies was the selective effects of DFMO on the polyamine levels of B-cells from Eμ-*Myc* mice. Specifically, DFMO effectively reduced putrescine levels, and to some extent spermidine content, back to those levels found in the B-cells of wild-type mice. By contrast, there was essentially no effect of DFMO on the polyamine levels of wild-type B-cells. Normal cells typically respond to DFMO treatment by increasing polyamine import, which is directed by a dedicated (but as yet uncharacterized) active transporter *(124)*. Strikingly, unlike its effect in normal cells, DFMO treatment *reduced* polyamine uptake in precancerous Eμ-*Myc* B-cells *(123)*. This finding suggests that precancerous cells that overexpress Myc have an Achilles' heel—they cannot recoup polyamines—and this explains why DFMO is so effective as a chemopreventative agent in cancer.

Mechanistically very few studies have addressed why Odc is required for tumorigenesis. DFMO studies in immortal myeloid cells had suggested that Odc was a mediator of Myc-induced apoptosis after the withdrawal of survival factors *(125)*. If this were universally true, then in the Eμ-*Myc* model DFMO treatment, and perhaps *Odc* heterozygosity, should impair Myc-induced apoptosis and be thus expected to accelerate disease, which is clearly the exact opposite of their effects *(123)*. Indeed, in vivo in primary B-cells having intact apoptotic checkpoints, DFMO and *Odc* heterozygosity did not affect Myc-induced apoptosis. Rather, the preventative effects of DFMO or *Odc* heterozygosity squarely centered on their ability to impair Myc's proliferative response *(123)*.

Myc accelerates the rates of cell cycle traverse, at least in part, by inhibiting the expression of the Cdk inhibitors p21^{Cip1} and p27^{Kip1}. This occurs at two levels, where Myc represses *p27^{Kip1}* promoter activity *(126)* and inhibits Miz-1-mediated transactivation of *p21^{Cip1}* *(75,79,82)*, and at the level of the protein, where Myc promotes degradation of p27^{Kip1} by the proteasome *(127–130)*. Interestingly, DFMO and *Odc* heterozygosity selectively blocked Myc's effects on p21^{Cip1} and p27^{Kip} protein, but not their RNA, levels *(123)*. Turnover of p21^{Cip1} and especially p27^{Kip1} protein is mediated by the SCF^{Skp2} complex that ubiquitylates these proteins to mark them for degradation

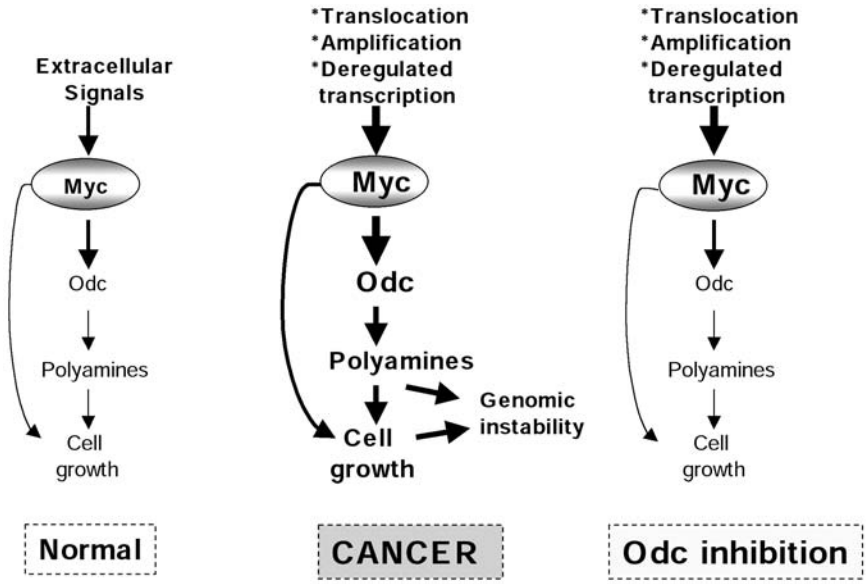

Fig. 5. Proposed mechanism of chemoprevention of Myc-induced cancers by targeting polyamine biosynthesis. In normal cells, Myc levels are tightly controlled, resulting in balanced rates of cell growth and proliferation. In various cancers, Myc levels are elevated as the consequence of mutations in upstream regulatory pathways or through direct genetic events involving *Myc* genes. This results in accelerated rates cell-cycle traverse, which puts the cell at risk for additional mutations and genomic instability. In precancerous cells, where one can perturb polyamine levels, Myc's ability to drive uncontrolled cell proliferation is hampered, which contributes to "genome-protection" and chemoprevention.

by the proteasome *(131–142)*, and $p27^{Kip1}$ loss (but not $p21^{Cip1}$ loss) markedly accelerates Myc-induced tumorigenesis in the Eμ-*Myc* mouse by selectively augmenting Myc's proliferative response *(143)*. These findings are thus consistent with a model where DFMO or *Odc* heterozygosity leads to a reduction in polyamine levels in Eμ-*Myc* B-cells that then somehow disrupts the functions of the SCF^{Skp2} complex. In turn, this leads to increases in these Cdk inhibitors and to the inhibition of cyclin-E/Cdk2 complexes and cell proliferation, which impair Myc-induced tumorigenesis (Fig. 5 and 6).

A second rather surprising finding coming from the analyses of Eμ-*Myc*; $Odc^{+/-}$ mice and DFMO-treated transgenics was that targeting this pathway also alters the route of transformation that usually accompanies lymphoma development *(123)*. A hallmark of Myc-induced malignancies is inactivation of the Arf-p53 tumor suppressor pathway, which is inactivated in most Burkitt lymphomas *(119,121)*; in the Eμ-*Myc* mouse, this involves biallelic deletions in *Arf* and missense "hot-spot" mutations in *p53* that generate dominant–negative forms of p53 protein *(118,120)*. Interestingly, lymphomas arising in Eμ-*Myc*;$Odc^{+/-}$ mice and in DFMO-treated transgenics selectively lacked deletions in

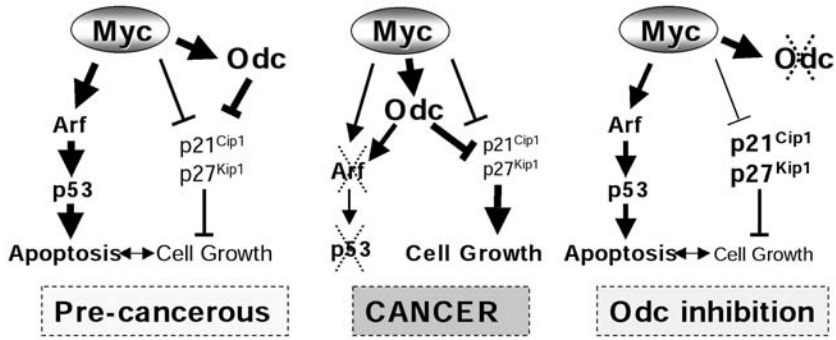

Fig. 6. Molecular events underlying prevention of Myc-induced lymphomagenesis by targeting Odc. In young precancerous Eμ-*Myc* transgenic animals, Myc induces the expression of Odc, which contributes to Myc's ability to suppress the expression of the Cdk inhibitors $p21^{Cip1}$ and $p27^{Kip1}$. In the absence of these cell cycle regulators, B-cells proliferate at an accelerated pace, which directly or indirectly (a matter of some debate), is sensed by the Arf/p53 tumor surveillance pathway. Over time, prolonged Myc expression results in cancer from mutations in the Arf/p53 pathway, which may be caused by the direct effect of polyamines on genomic instability or by replication errors inherent to the unchecked proliferative state. Therefore, inhibition of *Odc*, which reduces proliferative rates by effectively restoring proper expression of Cdk inhibitors and by reducing the production of polyamines, blocks mutations in the Arf/p53 tumor suppressor pathway, thereby preventing lymphomagenesis.

Arf *(123)*, suggesting that reductions in polyamine levels affect deletion/recombination events that are a hallmark of genomic instability *(144,145)* (Fig. 5 and 6). Furthermore, DFMO was ineffective in preventing lymphoma development in Eμ-*Myc* mice already bearing deletions in *Arf* or in rescuing animals with overt disease. These findings underscore the efficacy of DFMO as a chemopreventative, but not therapeutic, agent in cancer therapy and, importantly, indicate that targeting the polyamine pathway can prevent "second hits" that accompany the progression to overt malignancies.

3. Future Perspectives

Inhibitors of polyamine biosynthesis have largely been a disappointment as chemotherapeutic drugs *(116)* and we envision at least two reasons for their failure to show efficacy. First, cells respond to reductions in intracellular levels of polyamines by increasing polyamine uptake *(124)*, and we propose that polyamine uptake is robust in many tumor types, particularly in the case of advanced disease. Indeed, a role of polyamine transport in DFMO resistance has been elegantly demonstrated in mice in which L1210 tumor cells deficient in polyamine uptake are sensitive to therapeutic effects of DFMO vs parental L1210 cells that have efficient polyamine uptake *(146)*. Thus, in the Eμ-*Myc* system DFMO is effective as a preventive agent in the precancerous state where there are defects in polyamine uptake, yet it fails as a therapeutic with established disease, presumably from activation of the polyamine uptake pathway. Second, there is a genetic component in which multiple alterations that occur in cancer

bypass the effectiveness of inhibitors of polyamine biosynthesis, such as that which occurs in Eμ-*Myc* transgenics having preexisting mutations in *Arf*. Collectively, these hurdles limit the efficacy of such agents to early cancer chemoprevention, a notion now borne out in both preclinical and clinical studies *(116,147–152)*. If administered early on, the ability of such agents to harness rates of proliferation of the premalignant clone would markedly reduce the rates of occurrence of secondary hits that lead to overt transformation (Fig. 6).

Early diagnosis is essential for the proper use of agents in cancer chemoprevention because it is inappropriate to propose that an entire population should be on a drug. One success story in the clinic appears to be the use of DFMO in chemoprevention of colon cancer in which early diagnosis is indeed linked to successful outcome *(151,153,154)*, and here connections of the Myc-to-Odc pathway are clear, where Myc is deregulated by mutations that disable Wnt/β-catenin pathways *(23)*. However, other less studied avenues of chemoprevention are also likely appropriate, especially in cases of familial cancers having a high predisposition to the development of malignancies. These may include individuals from families harboring germline mutations in the tumor suppressors TP53 (Li-Fraumeni syndrome *[155]*), CHK2 (Li-Fraumeni-like syndrome *[156]*), or BRCA1 (familial breast cancer *[157]*). Although these conditions are rather rare, we would argue that clinical chemoprevention studies testing the efficacy of inhibitors of polyamine biosynthesis might show benefit, particularly given that secondary mutations are required for the development of overt malignancies in these patients. Another more common avenue for chemoprevention with such agents is perhaps in the prevention of relapse in various cancers, especially in regard to relapsed or secondary leukemia. DNA-damaging agents used in the clinic have the ability to efficiently kill tumors, and Myc-expressing cancer cells are especially sensitive to such agents *(158)*, but these treatments also generate *de novo* mutations in surviving cells that one might be able to prevent by using inhibitors of polyamine biosynthesis.

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References

1. Sheiness, D., Fanshier, L., and Bishop, J. M. (1978) Identification of nucleotide sequences which may encode the oncogenic capacity of avian retrovirus MC29. *J. Virol.* **28,** 600–610.
2. Sheiness, D. and Bishop, J. M. (1979) DNA and RNA from uninfected vertebrate cells contain nucleotide sequences related to the putative transforming gene of avian myelocytomatosis virus. *J. Virol.* **31,** 514–521.
3. Sheiness, D. K., Hughes, S. H., Varmus, H. E., Stubblefield, E., and Bishop, J. M. (1980) The vertebrate homolog of the putative transforming gene of avian myelocytomatosis virus: characteristics of the DNA locus and its RNA transcript. *Virology* **105,** 415–424.

4. Crews, S., Barth, R., Hood, L., Prehn, J., and Calame, K. (1982) Mouse c-myc oncogene is located on chromosome 15 and translocated to chromosome 12 in plasmacytomas. *Science* **218,** 1319–1321.
5. Dalla-Favera, R., Bregni, M., Erikson, J., Patterson, D., Gallo, R. C., and Croce, C. M. (1982) Human c-myc onc gene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. *Proc. Natl. Acad. Sci. USA* **79,** 7824–7827.
6. Kohl, N. E., Kanda, N., Schreck, R. R., et al. (1983) Transposition and amplification of oncogene-related sequences in human neuroblastomas. *Cell* **35,** 359–367.
7. Nau, M. M., Brooks, B. J., Battey, J., et al. (1985) L-myc, a new myc-related gene amplified and expressed in human small cell lung cancer. *Nature* **318,** 69–73.
8. Schwab, M., Varmus, H. E., Bishop, J. M, et al. (1984) Chromosome localization in normal human cells and neuroblastomas of a gene related to c-myc. *Nature* **308,** 288–291.
9. Charron, J., Malynn, B. A., Fisher, P., et al. (1992) Embryonic lethality in mice homozygous for a targeted disruption of the N-myc gene. *Genes Dev.* **6,** 2248–2257.
10. Davis, A. C., Wims, M., Spotts, G. D., Hann, S. R., and Bradley, A. (1993) A null c-myc mutation causes lethality before 10.5 days of gestation in homozygotes and reduced fertility in heterozygous female mice. *Genes Dev.* **7,** 671–682.
11. Moens, C. B., Auerbach, A. B., Conlon, R. A., Joyner, A. L., and Rossant, J. (1992) A targeted mutation reveals a role for N-myc in branching morphogenesis in the embryonic mouse lung. *Genes Dev.* **6,** 691–704.
12. Sawai, S., Shimono, A., Hanaoka, K., and Kondoh, H. (1991) Embryonic lethality resulting from disruption of both N-myc alleles in mouse zygotes. *New Biol.* **3,** 861–869.
13. Stanton, B. R., Reid, S. W., and Parada, L. F. (1990) Germ line transmission of an inactive N-myc allele generated by homologous recombination in mouse embryonic stem cells. *Mol. Cell Biol.* **10,** 6755–6758.
14. Hatton, K. S., Mahon, K., Chin, L., et al. (1996) Expression and activity of L-Myc in normal mouse development. *Mol. Cell Biol.* **16,** 1794–1804.
15. Baudino, T. A., McKay, C., Pendeville-Samain, H., et al. (2002) c-Myc is essential for vasculogenesis and angiogenesis during development and tumor progression. *Genes Dev.* **16,** 2530–43.
16. de Alboran, I. M., O'Hagan, R. C., Gartner, F., et al. (2001) Analysis of C-MYC function in normal cells via conditional gene-targeted mutation. *Immunity* **14,** 45–55.
17. Iritani, B. M. and Eisenman, R. N. (1999) c-Myc enhances protein synthesis and cell size during B lymphocyte development. *Proc. Natl. Acad. Sci. USA* **96,** 13,180–13,185.
18. Johnston, L. A., Prober, D. A., Edgar, B. A., Eisenman, R. N., and Gallant, P. (1999) Drosophila myc regulates cellular growth during development. *Cell* **98,** 779–790.
19. Knoepfler, P. S., Cheng, P. F., and Eisenman, R. N. (2002) N-myc is essential during neurogenesis for the rapid expansion of progenitor cell populations and the inhibition of neuronal differentiation. *Genes Dev.* **16,** 2699–2712.
20. Pelengaris, S., Littlewood, T., Khan, M., Elia, G., and Evan, G. (1999) Reversible activation of c-Myc in skin: induction of a complex neoplastic phenotype by a single oncogenic lesion. *Mol. Cell* **3,** 565–577.
21. Trumpp, A., Refaeli, Y., Oskarsson, T., et al. (2001) c-Myc regulates mammalian body size by controlling cell number but not cell size. *Nature* **414,** 768–773.
22. Duyao, M. P., Buckler, A. J., and Sonenshein, G. E. (1990) Interaction of an NF-kappa B-like factor with a site upstream of the c-myc promoter. *Proc. Natl. Acad. Sci. USA* **87,** 4727–4731.
23. He, T. C., Sparks, A. B., Rago, C., et al. (1998) Identification of c-MYC as a target of the APC pathway. *Science* **281,** 1509–1512.

24. Bromann, P. A., Korkaya, H., and Courtneidge, S. A. (2004) The interplay between Src family kinases and receptor tyrosine kinases. *Oncogene* **23,** 7957–7968.
25. Bahram, F., von der Lehr, N., Cetinkaya, C., and Larsson, L. G. (2000) c-Myc hot spot mutations in lymphomas result in inefficient ubiquitination and decreased proteasome-mediated turnover. *Blood* **95,** 2104–2110.
26. Bhatia, K., Huppi, K., Spangler, G., Siwarski, D., Iyer, R., and Magrath, I. (1993) Point mutations in the c-Myc transactivation domain are common in Burkitt's lymphoma and mouse plasmacytomas. *Nat. Genet.* **5,** 56–61.
27. Chou, T. Y., Dang, C. V., and Hart, G. W. (1995) Glycosylation of the c-Myc transactivation domain. *Proc. Natl. Acad. Sci. USA* **92,** 4417–4421.
28. Chou, T. Y., Hart, G. W., and Dang, C. V. (1995) c-Myc is glycosylated at threonine 58, a known phosphorylation site and a mutational hot spot in lymphomas. *J. Biol. Chem.* **270,** 18,961–18,965.
29. Gregory, M. A., Qi, Y., and Hann, S. R. (2003) Phosphorylation by glycogen synthase kinase-3 controls c-myc proteolysis and subnuclear localization. *J. Biol. Chem.* **278,** 51,606–51,612.
30. Lepique, A. P., Moraes, M. S., Rocha, K. M., et al. (2004) c-Myc protein is stabilized by fibroblast growth factor 2 and destabilized by ACTH to control cell cycle in mouse Y1 adrenocortical cells. *J. Mol. Endocrinol.* **33,** 623–638.
31. Luscher, B. and Eisenman, R. N. (1992) Mitosis-specific phosphorylation of the nuclear oncoproteins Myc and Myb. *J. Cell Biol.* **118,** 775–784.
32. Luscher, B., Kuenzel, E. A., Krebs, E. G., and Eisenman, R. N. (1989) Myc oncoproteins are phosphorylated by casein kinase II. *EMBO J.* **8,** 1111–1119.
33. Pulverer, B. J., Fisher, C., Vousden, K., Littlewood, T., Evan, G., and Woodgett, J. R. (1994) Site-specific modulation of c-Myc cotransformation by residues phosphorylated in vivo. *Oncogene* **9,** 59–70.
34. Saksela, K., Makela, T. P., Evan, G., and Alitalo, K. (1989) Rapid phosphorylation of the L-myc protein induced by phorbol ester tumor promoters and serum. *EMBO J.* **8,** 149–157.
35. Sears, R., Nuckolls, F., Haura, E., Taya, Y., Tamai, K., and Nevins, J. R. (2000) Multiple Ras-dependent phosphorylation pathways regulate Myc protein stability. *Genes Dev.* **14,** 2501–2514.
36. Yeh, E., Cunningham, M., Arnold, H., et al. (2004) A signalling pathway controlling c-Myc degradation that impacts oncogenic transformation of human cells. *Nat. Cell Biol.* **6,** 308–318.
37. Seeger, R. C., Brodeur, G. M., Sather, H., et al. (1985) Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas. *N. Engl. J. Med.* **313,** 1111–1116.
38. Cavalieri, F. and Goldfarb, M. (1987) Growth factor-deprived BALB/c 3T3 murine fibroblasts can enter the S phase after induction of c-myc gene expression. *Mol. Cell Biol.* **7,** 3554–3560.
39. Cavalieri, F. and Goldfarb, M. (1988) N-myc proto-oncogene expression can induce DNA replication in Balb/c 3T3 fibroblasts. *Oncogene* **2,** 289–291.
40. Askew, D. S., Ashmun, R. A., Simmons, B. C., and Cleveland, J. L. (1991) Constitutive c-myc expression in an IL-3-dependent myeloid cell line suppresses cell cycle arrest and accelerates apoptosis. *Oncogene* **6,** 1915–1922.
41. Evan, G. I., Wyllie, A. H., Gilbert, C. S., et al. (1992) Induction of apoptosis in fibroblasts by c-myc protein. *Cell* **69,** 119–128.
42. Debbas, M. and White, E. (1993) Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. *Genes Dev.* **7,** 546–554.

43. Lowe, S. W. and Ruley, H. E. (1993) Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. *Genes Dev.* **7,** 535–545.
44. Rao, L., Debbas, M., Sabbatini, P., Hockenbery, D., Korsmeyer, S., and White, E. (1992) The adenovirus E1A proteins induce apoptosis, which is inhibited by the E1B 19-kDa and Bcl-2 proteins. *Proc. Natl. Acad. Sci. USA* **89,** 7742–7746.
45. Kowalik, T. F., DeGregori, J., Schwarz, J. K., and Nevins, J. R. (1995) E2F1 overexpression in quiescent fibroblasts leads to induction of cellular DNA synthesis and apoptosis. *J. Virol.* **69,** 2491–2500.
46. Qin, X. Q., Livingston, D. M., Kaelin, W. G., Jr., and Adams, P. D. (1994) Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. *Proc. Natl. Acad. Sci. USA* **91,** 10,918–10,922.
47. Shan, B. and Lee, W. H. (1994) Deregulated expression of E2F-1 induces S-phase entry and leads to apoptosis. *Mol. Cell Biol.* **14,** 8166–8173.
48. Wu, X. and Levine, A. J. (1994) p53 and E2F-1 cooperate to mediate apoptosis. *Proc. Natl. Acad. Sci. USA* **91,** 3602–3606.
49. Nilsson, J. A. and Cleveland, J. L. (2003) Myc pathways provoking cell suicide and cancer. *Oncogene* **22,** 9007–9021.
50. Pelengaris, S., Khan, M., and Evan, G. (2002) c-MYC: more than just a matter of life and death. *Nat. Rev. Cancer* **2,** 764–776.
51. Prendergast, G. C. (1999) Mechanisms of apoptosis by c-Myc. *Oncogene* **18,** 2967–2987.
52. Ingvarsson, S., Asker, C., Axelson, H., Klein, G., and Sumegi, J. (1988) Structure and expression of B-myc, a new member of the myc gene family. *Mol. Cell Biol.* **8,** 3168–3174.
53. Sugiyama, A., Kume, A., Nemoto, K., et al. (1989) Isolation and characterization of s-myc, a member of the rat myc gene family. *Proc. Natl. Acad. Sci. USA* **86,** 9144–9148.
54. Ayer, D. E., Kretzner, L., and Eisenman, R. N. (1993) Mad: a heterodimeric partner for Max that antagonizes Myc transcriptional activity. *Cell* **72,** 211–222.
55. Hurlin, P. J., Queva, C., and Eisenman, R. N. (1997) Mnt, a novel Max-interacting protein is coexpressed with Myc in proliferating cells and mediates repression at Myc binding sites. *Genes Dev.* **11,** 44–58.
56. Hurlin, P. J., Queva, C., Koskinen, P. J., et al. (1995) Mad3 and Mad4: Novel Max-interacting transcriptional repressors that suppress c-myc dependent transformation and are expressed during neural and epidermal differentiation. *EMBO J.* **14,** 5646–5659.
57. Hurlin, P. J., Steingrimsson, E., Copeland, N. G., Jenkins, N. A., and Eisenman, R. N. (1999) Mga, a dual-specificity transcription factor that interacts with Max and contains a T-domain DNA-binding motif. *EMBO J.* **18,** 7019–7028.
58. Zervos, A. S., Gyuris, J., and Brent, R. (1993) Mxi1, a protein that specifically interacts with Max to bind Myc-Max recognition sites. *Cell* **72,** 223–232.
59. Zervos, A. S., Gyuris, J., and Brent, R. (1994) Mxi1, a protein that specifically interacts with Max to bind Myc-Max recognition sites. *Cell* **79,** 388.
60. Blackwood, E. M. and Eisenman, R. N. (1991) Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. *Science* **251,** 1211–1217.
61. Blackwood, E. M., Luscher, B., and Eisenman, R. N. (1992) Myc and Max associate in vivo. *Genes Dev.***6,** 71–80.
62. Blackwell, T. K., Huang, J., Ma, A., et al. (1993) Binding of myc proteins to canonical and noncanonical DNA sequences. *Mol. Cell Biol.* **13,** 5216–5224.
63. Blackwell, T. K., Kretzner, L., Blackwood, E. M., Eisenman, R. N., and Weintraub, H. (1990) Sequence-specific DNA binding by the c-Myc protein. *Science* **250,** 1149–1151.

64. Prendergast, G. C. and Ziff, E. B. (1991) Methylation-sensitive sequence-specific DNA binding by the c-Myc basic region. *Science* **251,** 186–189.
65. McMahon, S. B., Van Buskirk, H. A., Dugan, K. A., Copeland, T. D., and Cole, M. D. (1998) The novel ATM-related protein TRRAP is an essential cofactor for the c-Myc and E2F oncoproteins. *Cell* **94,** 363–374.
66. McMahon, S. B., Wood, M. A., and Cole, M. D. (2000) The essential cofactor TRRAP recruits the histone acetyltransferase hGCN5 to c-Myc. *Mol. Cell Biol.* **20,** 556–562.
67. Park, J., Kunjibettu, S., McMahon, S. B., and Cole, M. D. (2001) The ATM-related domain of TRRAP is required for histone acetyltransferase recruitment and Myc-dependent oncogenesis. *Genes Dev.* **15,** 1619–1624.
68. Dugan, K. A., Wood, M. A., and Cole, M. D. (2002) TIP49, but not TRRAP, modulates c-Myc and E2F1 dependent apoptosis. *Oncogene* **21,** 5835–5843.
69. Park, J., Wood, M. A., and Cole, M. D. (2002) BAF53 forms distinct nuclear complexes and functions as a critical c-Myc-interacting nuclear cofactor for oncogenic transformation. *Mol. Cell Biol.* **22,** 1307–1316.
70. Ayer, D. E., Laherty, C. D., Lawrence, Q. A., Armstrong, A. P., and Eisenman, R. N. (1996) Mad proteins contain a dominant transcription repression domain. *Mol. Cell Biol.* **16,** 5772–5781.
71. Ayer, D. E., Lawrence, Q. A., and Eisenman, R. N. (1995) Mad-Max transcriptional repression is mediated by ternary complex formation with mammalian homologs of yeast repressor Sin3. *Cell* **80,** 767–776.
72. Heinzel, T., Lavinsky, R. M., and Mullen, T. M, et al. (1997) A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression. *Nature* **387,** 43–48.
73. Nomura, T., Khan, M. M., Kaul, S. C., et al. (1999) Ski is a component of the histone deacetylase complex required for transcriptional repression by Mad and thyroid hormone receptor. *Genes Dev.* **13,** 412–423.
74. Bowen, H., Biggs, T. E., Phillips, E., et al. (2002) c-Myc represses and Miz-1 activates the murine natural resistance-associated protein 1 promoter. *J. Biol. Chem.* **277,** 34,997–35,006.
75. Herold, S., Wanzel, M., Beuger, V., et al. (2002) Negative regulation of the mammalian UV response by Myc through association with Miz-1. *Mol. Cell* **10,** 509–521.
76. Kime, L. and Wright, S. C. (2003) Mad4 is regulated by a transcriptional repressor complex that contains Miz-1 and c-Myc. *Biochem. J.* **370,** 291–298.
77. Oster, S. K., Marhin, W. W., Asker, C., et al. (2000) Myc is an essential negative regulator of platelet-derived growth factor beta receptor expression. *Mol. Cell Biol.* **20,** 6768–6778.
78. Peukert, K., Staller, P., Schneider, A., Carmichael, G., Hanel, F., and Eilers, M. (1997) An alternative pathway for gene regulation by Myc. *EMBO J.* **16,** 5672–5686.
79. Seoane, J., Le, H. V., and Massague, J. (2002) Myc suppression of the p21(Cip1) Cdk inhibitor influences the outcome of the p53 response to DNA damage. *Nature* **419,** 729–734.
80. Seoane, J., Pouponnot, C., Staller, P., Schader, M., Eilers, M., and Massague, J. (2001) TGFbeta influences Myc, Miz-1 and Smad to control the CDK inhibitor p15INK4b. *Nat. Cell Biol.* **3,** 400–408.
81. Staller, P., Peukert, K., Kiermaier, A., et al. (2001) Repression of p15INK4b expression by Myc through association with Miz-1. *Nat. Cell Biol.* **3,** 392–399.
82. Wu, S., Cetinkaya, C., Munoz-Alonso, M. J., et al. (2003) Myc represses differentiation-induced p21CIP1 expression via Miz-1-dependent interaction with the p21 core promoter. *Oncogene* **22,** 351–360.
83. Brenner, C., Deplus, R., Didelot, C., et al. (2005) Myc represses transcription through recruitment of DNA methyltransferase corepressor. *EMBO J.* **24,** 336–346.

84. Zeller, K. I., Jegga A. G., Aronow, B. J., O'Donnell, K. A., and Dang, C. V. (2003) An integrated database of genes responsive to the Myc oncogenic transcription factor: identification of direct genomic targets. *Genome Biol.* **4,** R69.
85. Boon, K., Caron H. N., van Asperen, R., et al. (2001) N-myc enhances the expression of a large set of genes functioning in ribosome biogenesis and protein synthesis. *EMBO J.* **20,** 1383–1393.
86. Coller, H. A., Grandori, C., Tamayo, P., et al. (2000) Expression analysis with oligonucleotide microarrays reveals that MYC regulates genes involved in growth, cell cycle, signaling, and adhesion. *Proc. Natl. Acad. Sci. USA* **97,** 3260–3265.
87. Ellwood-Yen, K., Graeber, T. G., Wongvipat, J., et al. (2003) Myc-driven murine prostate cancer shares molecular features with human prostate tumors. *Cancer Cell* **4,** 223–238.
88. Godfried, M. B., Veenstra, M., Sluis, P., et al. (2002) The N-myc and c-myc downstream pathways include the chromosome 17q genes nm23-H1 and nm23-H2. *Oncogene* **21,** 2097–2101.
89. Huang, E., Ishida, S., Pittman, J., et al. (2003) Gene expression phenotypic models that predict the activity of oncogenic pathways. *Nat. Genet.* **34,** 226–230.
90. Iritani, B. M., Delrow, J., Grandori, C., et al. (2002) Modulation of T-lymphocyte development, growth and cell size by the Myc antagonist and transcriptional repressor Mad1. *EMBO J.* **21,** 4820–4830.
91. Menssen, A. and Hermeking, H. (2002) Characterization of the c-MYC-regulated transcriptome by SAGE: identification and analysis of c-MYC target genes. *Proc. Natl. Acad. Sci. USA* **99,** 6274–6279.
92. Neiman, P. E., Ruddell, A., Jasoni, C., et al. (2001) Analysis of gene expression during myc oncogene-induced lymphomagenesis in the bursa of Fabricius. *Proc. Natl. Acad. Sci. USA* **98,** 6378–6383.
93. Watson, J. D., Oster, S. K., Shago, M., Khosravi, F., and Penn, L. Z. (2002) Identifying genes regulated in a Myc-dependent manner. *J. Biol. Chem.* **277,** 36,921–36,930.
94. Yu, Q., He, M., Lee, N. H., and Liu, E. T. (2002) Identification of Myc-mediated death response pathways by microarray analysis. *J. Biol. Chem.* **277,** 13,059–13,066.
95. Fernandez, P. C., Frank, S. R., Wang, L., et al. (2003) Genomic targets of the human c-Myc protein. *Genes Dev.* **17,** 1115–2119.
96. Haggerty, T. J., Zeller, K. I., Osthus, R. C., Wonsey, D. R., and Dang, C. V. (2003) A strategy for identifying transcription factor binding sites reveals two classes of genomic c-Myc target sites. *Proc. Natl. Acad. Sci. USA* **100,** 5313–5318.
97. Hulf, T., Bellosta, P., Furrer, M., et al. (2005) Whole-genome analysis reveals a strong positional bias of conserved dMyc-dependent e-boxes. *Mol. Cell Biol.* **25,** 3401–3410.
98. Mao, D. Y., Watson, J. D., Yan, P. S., et al. (2003) Analysis of Myc bound loci identified by CpG island arrays shows that Max is essential for Myc-dependent repression. *Curr. Biol.* **13,** 882–886.
99. O'Connell, B. C., Cheung, A. F., Simkevich, C. P., et al. (2003) A large scale genetic analysis of c-Myc-regulated gene expression patterns. *J. Biol. Chem.* **278,** 12,563–12,573.
100. Orian, A., van Steensel, B., Delrow, J., et al. (2003) Genomic binding by the Drosophila Myc, Max, Mad/Mnt transcription factor network. *Genes Dev.* **17,** 1101–1114.
101. Bush, A., Mateyak, M., Dugan, K., et al. (1998) c-myc null cells misregulate cad and gadd45 but not other proposed c-Myc targets. *Genes Dev.* **12,** 3797–3802.
102. Bello-Fernandez, C., Packham, G., and Cleveland, J. L. (1993) The ornithine decarboxylase gene is a transcriptional target of c-Myc. *Proc. Natl. Acad. Sci. USA* **90,** 7804–7808.
103. Pena, A., Reddy, C. D., Wu, S., et al. (1993) Regulation of human ornithine decarboxylase expression by the c-Myc.Max protein complex. *J. Biol. Chem.* **268,** 27,277–27,285.

104. Wagner, A. J., Meyers, C., Laimins, L. A., and Hay, N. (1993) c-Myc induces the expression and activity of ornithine decarboxylase. *Cell Growth Differ.* **4,** 879–883.
105. Pendeville, H., Carpino, N., Marine, J. C., et al. (2001) The ornithine decarboxylase gene is essential for cell survival during early murine development. *Mol. Cell Biol.* **21,** 6549–6558.
106. Nilsson, J. A., Maclean, K. H., Keller, U. B., Pendeville, H., Baudino, T. A., and Cleveland, J. L. (2004) Mnt loss triggers Myc transcription targets, proliferation, apoptosis, and transformation. *Mol. Cell Biol.* **24,** 1560–1569.
107. Auvinen, M., Jarvinen, K., Hotti, A, et al. (2003) Transcriptional regulation of the ornithine decarboxylase gene by c-Myc/Max/Mad network and retinoblastoma protein interacting with c-Myc. *Int. J. Biochem. Cell Biol.* **35,** 496–521.
108. Nilsson, J. A. and Cleveland, J. L. (2004) Mnt: Master regulator of the Max network. *Cell Cycle* **3,** 588–590.
109. Ayer, D. E. and Eisenman, R. N. (1993) A switch from Myc:Max to Mad:Max heterocomplexes accompanies monocyte/macrophage differentiation. *Genes Dev.* **7,** 2110–2119.
110. Hurlin, P. J., Ayer, D. E., Grandori, C., and Eisenman, R. N. (1994) The Max transcription factor network: Involvement of Mad in differentiation and an approach to identification of target genes. *Cold Spring Harb. Symp. Quant. Biol.* **59,** 109–116.
111. Pulverer, B., Sommer, A., McArthur, G. A., Eisenman, R. N., and Luscher, B. (2000) Analysis of Myc/Max/Mad network members in adipogenesis: inhibition of the proliferative burst and differentiation by ectopically expressed Mad1. *J. Cell. Physiol.* **183,** 399–410.
112. Queva, C., McArthur, G. A., Ramos, L. S., and Eisenman, R. N. (1999) Dwarfism and dysregulated proliferation in mice overexpressing the MYC antagonist MAD1. *Cell Growth Differ.* **10,** 785–796.
113. Hurlin, P. J., Queva, C., and Eisenman, R. N. (1997) Mnt: a novel Max-interacting protein and Myc antagonist. *Curr. Top. Microbiol. Immunol.* **224,** 115–121.
114. Hurlin, P. J., Zhou, Z. Q., Toyo-Oka, K., et al. (2003) Deletion of Mnt leads to disrupted cell cycle control and tumorigenesis. *EMBO J.* **22,** 4584–4596.
115. Walker, W., Zhou, Z. Q., Ota, S., Wynshaw-Boris, A., and Hurlin, P. J. (2005) Mnt-Max to Myc-Max complex switching regulates cell cycle entry. *J. Cell Biol.* **169,** 405–413.
116. Gerner, E. W. and Meyskens, F. L., Jr. (2004) Polyamines and cancer: old molecules, new understanding. *Nat. Rev. Cancer* **4,** 781–792.
117. Adams, J. M., Harris, A. W., Pinkert, C. A., et al. (1985) The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. *Nature* **318,** 533–538.
118. Eischen, C. M., Weber, J. D., Roussel, M. F., Sherr, C. J., and Cleveland, J. L. (1999) Disruption of the ARF-Mdm2-p53 tumor suppressor pathway in Myc-induced lymphomagenesis. *Genes Dev.* **13,** 2658–2669.
119. Lindstrom, M. S., Klangby, U., and Wiman, K. G. (2001) p14ARF homozygous deletion or MDM2 overexpression in Burkitt lymphoma lines carrying wild type p53. *Oncogene* **20,** 2171–2177.
120. Schmitt, C. A., McCurrach, M. E., de Stanchina, E., Wallace-Brodeur, R. R., and Lowe, S. W. (1999) INK4a/ARF mutations accelerate lymphomagenesis and promote chemoresistance by disabling p53. *Genes Dev.* **13,** 2670–2677.
121. Wilda, M., Bruch, J., Harder, L., et al. (2004) Inactivation of the ARF-MDM-2-p53 pathway in sporadic Burkitt's lymphoma in children. *Leukemia* **18,** 584–588.
122. Jacobsen, K. A., Prasad, V. S., Sidman, C. L., and Osmond, D. G. (1994) Apoptosis and macrophage-mediated deletion of precursor B cells in the bone marrow of E mu-myc transgenic mice. *Blood* **84,** 2784–2794.

123. Nilsson, J. A., Keller, U., Baudino, T. A., et al. (2005) Targeting ornithine decarboxylase in Myc-induced lymphomagenesis prevents tumor formation. *Cancer Cell* **7,** 433–444.
124. Seiler, N., Delcros, J. G., and Moulinoux, J. P. (1996) Polyamine transport in mammalian cells. An update. *Int. J. Biochem. Cell Biol.* **28,** 843–861.
125. Packham, G. and Cleveland, J. L. (1994) Ornithine decarboxylase is a mediator of c-Myc-induced apoptosis. *Mol. Cell Biol.* **14,** 5741–5747.
126. Yang, W., Shen, J., Wu, M., et al. (2001) Repression of transcription of the p27(Kip1) cyclin-dependent kinase inhibitor gene by c-Myc. *Oncogene* **20,** 1688–1702.
127. Alevizopoulos, K., Vlach, J., Hennecke, S., and Amati, B. (1997) Cyclin E and c-Myc promote cell proliferation in the presence of p16INK4a and of hypophosphorylated retinoblastoma family proteins. *EMBO J.* **16,** 5322–5333.
128. Amati, B. and Vlach, J. (1999) Kip1 meets SKP2: new links in cell-cycle control. *Nat. Cell Biol.* **1,** E91–E93.
129. O'Hagan, R. C., Ohh, M., David, G., et al. (2000) Myc-enhanced expression of Cul1 promotes ubiquitin-dependent proteolysis and cell cycle progression. *Genes Dev.* **14,** 2185–2191.
130. Vlach, J., Hennecke, S., Alevizopoulos, K., Conti, D., and Amati, B. (1996) Growth arrest by the cyclin-dependent kinase inhibitor p27Kip1 is abrogated by c-Myc. *EMBO J.* **15,** 6595–6604.
131. Bashir, T., Dorrello, N. V., Amador, V., Guardavaccaro, D., and Pagano, M. (2004) Control of the SCF(Skp2-Cks1) ubiquitin ligase by the APC/C(Cdh1) ubiquitin ligase. *Nature* **428,** 190–193.
132. Bornstein, G., Bloom, J., Sitry-Shevah, D., Nakayama, K., Pagano, M., and Hershko, A. (2003) Role of the SCFSkp2 ubiquitin ligase in the degradation of p21Cip1 in S phase. *J. Biol. Chem.* **278,** 25,752–25,757.
133. Elledge, S. J. and Harper, J. W. (1998) The role of protein stability in the cell cycle and cancer. *Biochim. Biophys. Acta* **1377,** M61–M70.
134. Ganoth, D., Bornstein, G., Ko, T. K., et al. (2001) The cell-cycle regulatory protein Cks1 is required for SCF(Skp2)-mediated ubiquitinylation of p27. *Nat. Cell Biol.* **3,** 321–324.
135. Kossatz, U., Dietrich, N., Zender, L., Buer, J., Manns, M. P., and Malek, N. P. (2004) Skp2-dependent degradation of p27kip1 is essential for cell cycle progression. *Genes Dev.* **18,** 2602–2607.
136. Morimoto, M., Nishida, T., Honda, R., and Yasuda, H. (2000) Modification of cullin-1 by ubiquitin-like protein Nedd8 enhances the activity of SCF(skp2) toward p27(kip1). *Biochem. Biophys. Res. Commun.* **270,** 1093–1096.
137. Nakayama, K., Nagahama, H., Minamishima, Y. A., et al. (2000) Targeted disruption of Skp2 results in accumulation of cyclin E and p27(Kip1), polyploidy and centrosome overduplication. *EMBO J.* **19,** 2069–2081.
138. Sitry, D., Seeliger, M. A., Ko, TK, et al. (2002) Three different binding sites of Cks1 are required for p27-ubiquitin ligation. *J. Biol. Chem.* **277,** 42,233–42,240.
139. Tsvetkov, L. M., Yeh, K. H., Lee, S. J., Sun, H., and Zhang, H. (1999) p27(Kip1) ubiquitination and degradation is regulated by the SCF(Skp2) complex through phosphorylated Thr187 in p27. *Curr. Biol.* **9,** 661–664.
140. Wei, W., Ayad, N. G., Wan, Y., Zhang, G. J., Kirschner, M. W., and Kaelin, W. G., Jr. (2004) Degradation of the SCF component Skp2 in cell-cycle phase G1 by the anaphase-promoting complex. *Nature* **428,** 194–198.
141. Yu, Z. K., Gervais, J. L., and Zhang, H. (1998) Human CUL-1 associates with the SKP1/SKP2 complex and regulates p21(CIP1/WAF1) and cyclin D proteins. *Proc. Natl. Acad. Sci. USA* **95,** 11,324–11,329.

142. Zheng, N., Schulman, B. A., Song, L., et al. (2002) Structure of the Cul1-Rbx1-Skp1-F boxSkp2 SCF ubiquitin ligase complex. *Nature* **416,** 703–709.
143. Martins, C. P., and Berns, A. (2002) Loss of p27(Kip1) but not p21(Cip1) decreases survival and synergizes with MYC in murine lymphomagenesis. *EMBO J.* **21,** 3739–3748.
144. Motoyama, N. and Naka, K. (2004) DNA damage tumor suppressor genes and genomic instability. *Curr. Opin. Genet. Dev.* **14,** 11–16.
145. Rajagopalan, H. and Lengauer, C. (2004) Aneuploidy and cancer. *Nature* **432,** 338–341.
146. Persson, L., Holm, I., Ask, A., and Heby, O. (1988) Curative effect of DL-2-difluoromethylornithine on mice bearing mutant L1210 leukemia cells deficient in polyamine uptake. *Cancer Res.* **48,** 4807–4811.
147. Einspahr, J. G., Bowden, G. T., and Alberts, D. S. (2003) Skin cancer chemoprevention: strategies to save our skin. *Recent Results Cancer Res.* **163,** 151–164; discussion 264–266.
148. Elmore, E., Stringer, D. E., Steele, V. E., Gerner, E. W., and Redpath, J. L. (2001) Chemoprevention by difluoromethylornithine: correlation of an in vitro human cell assay with human clinical data for biomarker modulation. *Anticancer Res.* **21,** 1163–1165.
149. Fabian, C. J. and Kimler, B. F. (2001) Beyond tamoxifen new endpoints for breast cancer chemoprevention, new drugs for breast cancer prevention. *Ann. NY Acad. Sci.* **952,** 44–59.
150. Kamat, A. M. and Lamm, D. L. (2002) Chemoprevention of bladder cancer. *Urol. Clin. North Am.* **29,** 157–168.
151. Umar, A., Viner, J. L., and Hawk, E. T. (2001) The future of colon cancer prevention. *Ann. NY Acad. Sci.* **952,** 88–108.
152. Wallace, H. M. and Fraser, A. V. (2003) Polyamine analogues as anticancer drugs. *Biochem. Soc. Trans.* **31,** 393–396.
153. Jacoby, R. F., Cole, C. E., Tutsch, K., et al. (2000) Chemopreventive efficacy of combined piroxicam and difluoromethylornithine treatment of Apc mutant Min mouse adenomas, and selective toxicity against Apc mutant embryos. *Cancer Res.* **60,** 1864–1870.
154. Erdman, S. H., Ignatenko, N. A., Powell, M. B., et al. (1999) APC-dependent changes in expression of genes influencing polyamine metabolism, and consequences for gastrointestinal carcinogenesis, in the Min mouse. *Carcinogenesis* **20,** 1709–1713.
155. Herbert, B. S., Wright, A. C., Passons, C. M., et al. (2001) Effects of chemopreventive and antitelomerase agents on the spontaneous immortalization of breast epithelial cells. *J. Natl. Cancer Inst.* **93,** 39–45.
156. Vahteristo, P., Tamminen, A., Karvinen, P., et al. (2001) p53, CHK2, and CHK1 genes in Finnish families with Li-Fraumeni syndrome: further evidence of CHK2 in inherited cancer predisposition. *Cancer Res.* **61,** 5718–5722.
157. Klemp, J., Brady, D., Frank T. S., Kimler, B. F., and Fabian, C. J. (2000) Incidence of BRCA1/2 germ line alterations in a high risk cohort participating in a phase II chemoprevention trial. *Eur. J. Cancer* **36,** 1209–1214.
158. Maclean, K. H., Keller, U. B., Rodriguez-Galindo, C., Nilsson, J. A., and Cleveland, J. L. (2003) c-Myc Augments Gamma Irradiation-Induced Apoptosis by Suppressing Bcl-X(L). *Mol. Cell Biol.* **23,** 7256–7270.

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Protective Effect of Polyamines on NSAID-Induced Injury and Apoptosis

Heather M. Wallace and Alun Hughes

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most commonly used drugs worldwide with more than 50 different agents on the market. They are used routinely for minor aches and pains, fever, and as anti-inflammatory drugs. More recently, aspirin has also been used as a chemopreventative agent in patients with cardiovascular disease as secondary prevention against stroke and myocardial infarction, and as a primary preventative agent in individuals at risk of developing colorectal cancer.

Despite their utility, NSAIDs exhibit a poor safety profile, with as many as 25% of all adverse drug reactions reported in the United Kingdom per annum involving NSAID use. The pharmacological activity of the NSAIDs is attributed to inhibition of the cyclooxygenase enzymes (COX-1 and 2). The gastrointestinal (GI) side effects associated with NSAID use are often severe and polyamines play a protective role against injury in the gut, as well as promoting efficient wound healing.

The mechanism through which the NSAIDs exert their chemopreventative effect is less clearly understood. Several groups, including our laboratory, have shown that the cytotoxic effects on tumor cells appear to be independent of COX inhibition, thus another mechanism must be involved. Our studies suggest the polyamine pathway is also a target for inhibition by NSAIDs. Thus the antiproliferative effects of NSAIDs may be mediated through loss of the polyamines that are known to be essential for cell and tumor growth. This chapter examines the protective roles of the polyamines against NSAID-induced injury in the gut and the broader implications of polyamine involvement in NSAID-induced apoptosis in tumor cells.

2. Background

It has been known for centuries that willow bark has therapeutic properties; however, it was not until 1860 that the active principle, salicylic acid, was first synthesized chemically *(1)*. Acetylsalicylic acid, commonly known as aspirin, was first introduced

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as a drug in 1899 by the Bayer Company, but its mechanism of action was not elucidated until 1971 when Sir John Vane's group showed that aspirin and other similar compounds inhibited prostaglandin (PG) biosynthesis *(2)*. These drugs were all found to possess anti-inflammatory, analgesic, and antipyretic properties, but their structures lacked steroid groups. This led to the broad classification of this loosely related family of agents as the NSAIDs.

NSAIDs are among the most commonly used drugs worldwide. They are used intermittently to treat mild-to-moderate pain and chronically to treat inflammatory conditions, such as rheumatoid arthritis. With more than 50 different NSAIDs on the market the scope for use, potential use, and profit is enormous.

The therapeutic mechanism of action of the NSAIDs is through inhibition of COX and the subsequent prevention of PG biosynthesis. There are two forms of cyclo-oxygenase, namely COX-1 and COX-2. COX-1 is the constitutive form of the enzyme *(3)* expressed on the endoplasmic reticulum of all cells *(4)*. The *COX-1* gene is located on chromosome 9 and is 22-kb long *(5)*. *COX-1* is generally considered a housekeeping gene with a role in platelets, the stomach, the kidney, and endothelial cells. *COX-2*, on the other hand, is not expressed constitutively except in the brain, but is rapidly induced (1–3 h) in response to mediators of inflammation, growth factors, and tumor promoters *(5,6)*. The *COX-2* gene is present on chromosome 1 and is 8 kb in size. *COX-2* is associated with the inflammatory response and has also been associated with the development of malignant disease from overexpression of *COX* in a variety of solid tumors, including colorectal cancer *(6–10)*.

2.1. Adverse Reactions to NSAIDs

Despite their therapeutic value, NSAIDs are known to induce severe GI side effects, including lesions and ulceration of the stomach. Aspirin is considered to be a high-risk drug for gastric injury, with naproxen being classed as moderate, sulindac as low, and nabumetone as very low risk *(11)*. Typically, a 500-mg daily dose of naproxen is associated with a 5% occurrence of gastric ulcers, whereas sulindac (300 mg daily) carries about a 1% risk. The incidence of less severe gastric erosions is, however, much higher especially with the more commonly used NSAIDs such as aspirin. Ibuprofen (2.4 g daily) and aspirin (2.6 g daily) have been shown to induce lesions in 85 and 94% of patients respectively within 7 d of treatment *(12)*. Opinions vary as to how many hospitalizations or deaths are due directly to ulcerations and bleeding attributed to NSAID use, but it is estimated that there are 3500 hospitalizations and 400 deaths per annum in the United Kingdom in those age 60 and older *(13)*.

There is a strong correlation between the selectivity ratio of inhibition of COX isoenzymes (COX-1:COX-2) and the severity of the GI side effects (Table 1); the higher the ratio, the more severe the side effects *(14)*. In terms of the structure of COX, selectivity of the binding site for NSAIDs on either isoenzyme appears to differ by a single amino acid. Substitution at position 509 of isoleucine to valine can reverse the apparent selectivity of COX-2-specific inhibitors *(15)*.

In an attempt to retain the beneficial therapeutic effects of the NSAIDs while decreasing the associated side effects, two new classes of NSAIDs have been developed

recently. The first is the selective COX-2 inhibitors designed to inhibit only the isoform of COX involved in inflammation and disease (namely COX-2) and include drugs such as celecoxib and nimesulide *(16)*. Despite improvements in GI tolerability, most selective COX-2 inhibitors display the other classical NSAID side effects, such as sodium and water retention, hypertension, and edema caused by COX-2 inhibition *(13)*.

The COX-2 selective inhibitors are of similar efficacy to the nonselective drugs in terms of relief of symptoms. However, they were marketed on the basis of inducing fewer side effects. The majority of clinical data supporting the use of COX-2 selective inhibitors comes from the Celecoxib Long-term Arthritis Safety Study trial *(17)*. This trial was carried out over 2 yr (1998–2000) and involved 8059 patients with osteoarthritis or rheumatoid arthritis. Despite the success in preventing GI side effects, other unpredictable cardiovascular side effects have resulted in the withdrawal of rofecoxib (Vioxx) and the instigation of Food and Drug Administration hearings into the safety profile of the entire class of selective COX-2 inhibitors.

A second novel class of NSAIDs was designed on the principle of incorporating a nitric oxide (NO)-donating moiety into the existing NSAID structure. Because both NO and PGs (the products of the COX enzyme) are essential for the maintenance of mucosal integrity, the theory is that NO released from the NO-NSAID will act as a protective agent against the side effects of the NSAIDs *(18)*. Interestingly, the synthesis of NO and the polyamines are linked through L-arginine, with the amino acid being the point of divergence of the two synthetic pathways. The NO-NSAIDs are not licensed for use in man, but NO-donating conjugates with NSAIDs, such as indomethacin, ibuprofen, and aspirin are undergoing the late stages of clinical testing.

Other strategies employed to decrease the risk of lesions and ulcers with NSAID use include the use of a combination of lower risk NSAIDs (including the selective COX-2 inhibitors) together with misoprostol, a PG analog, or proton pump inhibitors (omeprazole) and avoiding agents which potentiate lesions, such as H_2 receptor antagonists *(13)*.

3. The Role of the PGs

PGs, the products of COX activity, are present in relatively large amounts in the gastrointestinal mucosa *(19)*. Disruption of the homeostatic production of PGs by COX-1 inhibition should generally, therefore, be avoided. This is difficult because of the lack of selectivity of the range of NSAIDs available at present. However, there is evidence that dual inhibition of both isoforms of COX is required for the NSAIDs to induce GI lesions. Wallace et al. *(20)* demonstrated in rat models that although selective inhibition of COX-1 did decrease PG production significantly in the gut, it did not induce lesions *(20)*. Furthermore, ketorolac (which has far greater selectivity for COX-1 than COX-2) does not induce lesions at concentrations sufficient to inhibit only COX-1, but does cause damage at higher concentrations affecting both isoforms *(21)*.

As NSAIDs are nonspecific inhibitors of both isoforms of COX they inhibit all PG production, including that which is necessary for cell homeostasis. Maintenance of PG levels is important for cell proliferation and efficient wound healing. The PGs also play an important cytoprotective role in the gut preventing damage induced by aberrant acid

production *(22)*. Inhibition of PG synthesis by the NSAIDs negates this protective effect, leading to increased acid production and potentiation of GI damage. PGs are protective not only in the upper GI tract, but also through and into the small intestine *(23)*. Their protective effects against acid intolerance diminishes in the large intestine, where the environment is generally more pH neutral *(22)*.

The toxicity of the NSAIDs is not limited to the effects of inhibition of PG synthesis. They also display several topical sites of mucosal irritation, including uncoupling of mitochondrial respiration and osmotic lysis of epithelial cells in the stomach and duodenum caused by intracellular accumulation of ionized drugs *(24)*. The extent to which these topical sites play a role in the induction of erosions is debatable. Because exogenous prostaglandins can protect adequately against NSAID toxicity, they may have a less important role in ulceration.

4. Resolution of GI Injury

Regardless of the mechanisms of toxicity, the epithelial cells of the stomach are well adapted to healing the frequent superficial injuries obtained during exposure to toxic agents, such as the NSAIDs. There are two main mechanisms that operate to heal these minor erosions, the first is termed "restitution" and involves the rapid migration of unaffected epithelial cells from the surrounding area to cover the affected region. The secondary event (occurring 12–16 h later) is the restoration of the mucosal thickness and involves cell proliferation. Restitution requires the rapid flattening, spread, migration, and repolarization of differentiated columnar epithelial cells to cover the denuded area, but is independent of proliferation. Epithelial cells migrate from the adjacent unaffected area by the extension of lamellipodia and filopodia. These protrusions are similar to those used during macrophage and fibroblast migration and are rich in F-actin and myosin, both of which are essential for maintaining the structure and motility essential for the wound healing process *(25)*. Through the formation of lamellipodia and filopodia, epithelial cells contract and move toward focal adhesions *(26)*. It is well-known that the NSAIDs, in addition to inducing lesions, prevent the healing of denuded stomach regions both by inhibiting or delaying restitution and inhibiting the later events of cell proliferation. One way in which NSAIDs might prevent restitution is by inhibition of actin stress fiber formation and focal adhesion kinase (FAK) preventing epithelial cells from adhering to focal adhesion points, thus inhibiting cell migration *(26)*.

The polyamines, although more commonly associated with proliferative events, are essential for many phases of restitution and their relative abundance within the cell; the extracellular environment enables them to play a significant role in the efficient resealing of superficial lesions in response to GI perturbing drugs, such as the NSAIDs. Restitution is associated both in vitro and in vivo with a significant increase in polyamine biosynthesis *(27–29)*. Ornithine decarboxylase (ODC) activity and the maintenance of polyamine content are also necessary for the proliferative response to injury that occurs after restitution to restore the mucosal layer thickness *(30)*. As mentioned earlier, we and others have demonstrated the ability of the NSAIDs to induce toxicity in vitro in GI-related cancer cells that is linked to depletion of intracellular polyamines

and inhibition of ODC *(31–33)*. Furthermore, inhibition of ODC by α-difluoromethylornithine (DFMO) delayed the recovery of rat mucosal damage induced by arabinosylcytosine *(30)*. This observation was confirmed in stress-induced lesions in rat duodenal mucosa, in which increased ODC activity and polyamine content were observed during the healing process. In addition, inhibition of ODC and the subsequent polyamine depletion by DFMO prevented the healing process *(34)*. Because NSAIDs inhibit polyamine biosynthesis *(32)*, it may be that polyamine depletion contributes to the cellular damage induced by the NSAIDs by preventing wound healing through inhibition of both restitution and epithelial cell proliferation.

Clearly, because polyamines are essential for maintenance of normal cell growth and act as both growth factors and cell-cycle regulators, it is clear that their depletion would lead to retardation of the secondary proliferative response during wound healing. Less obvious is the polyamine's involvement in restitution. Extensive work by Wang et al. has demonstrated that the polyamines may influence restitution by alteration of the cytosolic concentration of calcium ions and thereby influence contractility and migration, whereas other work by Banan et al. has demonstrated that the polyamines may directly affect the polymerization and organization of microtubules affecting the cytoskeletal arrangement of the cells *(28,29,35–37)*.

In the same way as polyamines form a convergence point for cell proliferation, the cytosolic concentration of calcium ions ($[Ca^{2+}]_{cyt}$) forms a convergence point for the contraction and migration events of restitution, causing the mobilization of myosin along the actin filament framework and stimulating migration through activation of Ca^{2+}/calmodulin-dependent kinase II. $[Ca^{2+}]_{cyt}$ is controlled both by the influx of Ca^{2+} ions across the cell membrane and by release from intracellular stores. The passage of Ca^{2+} ions into the cell depends on electrochemical gradient from the external environment to the intracellular compartment and in nonexcitable cells (e.g., epithelial cells) this gradient is governed by the membrane potential not L-type voltage-dependent Ca^{2+} channels. Depolarization of the membrane leads to a decreased driving force resulting in less Ca^{2+} influx. Conversely, hyperpolarization favors influx and a corresponding increase in $[Ca^{2+}]_{cyt}$. In epithelial cells, the main contributing factor to the membrane potential is potassium ion permeability and the corresponding K^+ gradient formed across the plasma membrane. In cells that do not express voltage-dependent Ca^{2+} channels, voltage gated K^+ channels (Kv) regulate the membrane potential, with an increase in opening or number of Kv channels leading to hyperpolarization, causing Ca^{2+} influx and an increase in $[Ca^{2+}]_{cyt}$. Increased $[Ca^{2+}]_{cyt}$ then favors the contraction and migration of cells essential for the restitution response *(28,29,35,36)*.

The polyamines are able to alter the influx of Ca^{2+} into the cell by modulating Kv channels and also regulating their expression, thus altering the important $[Ca^{2+}]_{cyt}$ in favor of restitution events. Much of this work has been demonstrated in the IEC-6 cell system, an intestinal epithelial cell system that responds to monolayer wounding in vitro in a similar way to restitution in vivo. Polyamine depletion in IEC-6 cells by treatment with DFMO causes a decrease in the expression of mRNA for the Kv channel α-subunit, Kv1.1, to 5% of control. This effect is reversed completely by the addition of exogenous spermidine or putrescine, indicating that polyamines are essential

for the expression of functional Kv channels in epithelial cells. Furthermore, depletion of polyamines resulted in membrane depolarization and a subsequent drop in $[Ca^{2+}]_{cyt}$. The membrane potential was restored and the decrease in $[Ca^{2+}]_{cyt}$ reversed by the addition of spermidine. The absolute involvement of Ca^{2+} influx was confirmed by the failure of polyamines alone to reverse the membrane depolarization and restore restitution in IEC-6 cells depleted of both polyamines and extracellular Ca^{2+} *(28)*. Changes in the membrane potential induced by polyamine depletion and readdition indicate that as well as regulating the expression of Kv channels, the polyamines may also modulate their activity *(29)*.

It is clear that the polyamines can regulate voltage gated K^+ current sufficiently to induce changes in membrane polarization and effect $[Ca^{2+}]_{cyt}$ and ultimately aid efficient restitution. A key factor in restitution is the mobility of the cells that are reliant on the interaction of actin and myosin to cause contractile movement in the lamellipodia and filopodia extensions, as well as the retraction of the tailing side of the cell. Polyamines increase $[Ca^{2+}]_{cyt}$ causing an increase in the activity of the small guanosine 5′-triphosphatase RhoA, which in turn activates Rho-kinase causing myosin light chains phosphorylation *(29,38)*. Myosin light chain phosphorylation is a vital component of cell migration, essential for both actin–myosin interactions and the formation of stress fibers responsible for cell motility. Tyrosine phosphorylation of β-catenin is also affected by polyamine-mediated changes in $[Ca^{2+}]_{cyt}$ leading to alterations in cell-to-cell contact. β-catenin is associated with cell–cell adhesion via interaction with E-cadherin, but it can also induce gene expression by translocation to the nucleus where it acts as a transcriptional regulator. An increase in $[Ca^{2+}]_{cyt}$ caused by the polyamines increases tyrosine phosphorylation of β-catenin, which decreases its affinity for E-cadherin, initiating dissociation of cell-to-cell contact and promoting epithelial cell disassembly *(36)*. Presumably, changes in the phosphorylation of β-catenin caused by the polyamines alters the actions of β-catenin as a transcriptional regulator, which may have effects on restitution and later stage proliferation, but this remains to be investigated. Similarly, polyamines also cause changes in the tyrosine phosphorylation of FAK, leading to decreased attachment of cells to focal adhesion points when polyamine content is decreased *(39)*. As discussed earlier, FAK is inhibited by the NSAIDs leading to impairment of the wound healing process *(26)*, but it is not known if this is mediated through alterations in polyamine content or by another mechanism.

Restitution is a complicated multifactorial process and the mechanisms mediated through $[Ca^{2+}]_{cyt}$ represent just one of many contributing pathways to efficient wound healing. The polyamines have also been shown to affect microtubule formation as well as the activity of the adhesion related FAK both of which may contribute to polyamine regulation of wound healing in response to agents, such as the NSAIDs *(37,39)*. The effects of polyamines upon the polymerization and organization of microtubules observed by Banan et al. may result from electrostatic interactions within the cell aiding assembly of the cytoskeletal structure required for migration. On the other hand, polyamine depletion is known to negatively regulate the expression of genes associated with the cytoskeleton, including α-tubulin *(40)*. This may indicate that the polyamines act during restitution to physically aid assembly of the cytoskeleton while also being

involved in the longer term maintenance of migration and cell structure through gene expression. Because polyamine depletion in the above models causes inhibition of the restitution process and because NSAIDs have been shown to decrease intracellular polyamine content, it is logical to suggest that NSAIDs may inhibit wound healing through interaction with polyamine-dependent pathways of restitution. Indeed, polyamine depletion also sensitizes epithelial cells to the growth inhibitory effects of transforming growth factor-β, further suggesting that depletion within the gut will prevent both restitution and the efficient proliferative response involved in restoring mucosal layer thickness *(41)*.

5. Chemoprevention

Chemoprevention is the use of chemical agents, including drugs or food supplements, to prevent disease. The use of NSAIDs has essentially been rejuvenated by the discovery that they can prevent both secondary complications arising from cardiovascular disease and colorectal cancer in man. For the purposes of this chapter, we will focus on the use of NSAIDs as primary preventative agents in cancer. The use of NSAIDs in cardiovascular disease has been reviewed elsewhere *(42)*.

A number of case-controlled and cohort studies have shown that NSAIDs decrease the incidence of colorectal cancer in man by as much as 50% *(43)*. This effect requires the regular use (5–10 yr) of NSAIDs and it appears that NSAIDs prevent both initiation and promotion events *(43,44)*. Recent studies suggest that there is a positive correlation between the dose of the NSAID and the protection offered. This must, however, be balanced with the fact that increasing the dose of NSAID will also increase the incidence of side effects.

In vitro studies have shown that the NSAIDs induce apoptosis in cancer cells, but how this is achieved is less clear. Logically, one would assume that the growth inhibitory/cytotoxic effects of the NSAIDs were attributable to the inhibition of the COX enzymes. However, a large body of evidence now exists suggesting that this is not the case. It has been demonstrated on numerous occasions that the NSAIDs are capable of inducing apoptosis in colorectal cancer cells in culture, including some that do not express either isoform of COX *(45)*. In our laboratory, we have shown that NSAIDs were effective cytotoxic agents in a variety of tumor cells in culture (including colorectal, bladder, and breast) preventing cell proliferation with IC_{50} values being similar in most cell types (Table 2). Concomitant with the inhibition of cell growth we observed a decrease in intracellular polyamine content of 40 to 75%. This depletion was found to be a combination of inhibition of ODC, induction of spermidine/spermine N^1-acetyltransferase (SSAT) and polyamine oxidase (PAO), together with increased polyamine export (*[32]*; Wallace et al., unpublished observations). Arguably, these effects are seen in response to a variety of growth inhibitors *(46)* due to the requirement for polyamines for cell proliferation. On the other hand, polyamine depletion is known to sensitize various cell types to apoptosis and induce caspase-3. In addition, oxidation of polyamines by PAO generates hydrogen peroxide, which, in turn, can induce local apoptosis *(47)*. Hydrogen peroxide can also induce the activity of SSAT, the enzyme that generates the acetylpoyamines, which in turn are substrates for PAO thus creating a self perpetuating “death-inducing”

Table 1
COX Selectivity of Common NSAIDs

NSAID	COX-1/COX-2 ratio
Aspirin	0.01
Salicylate	1.00
Ibuprofen	0.07
Naproxen	0.88
Sulindac	0.61
Indomethacin	0.02

NSAID, nonsteroidal anti-inflammatory drugs; COX, cyclo-oxygenase.

Table 2
Cytotoxicity of Nonsteroidal Anti-Inflammatory Drugs in Human Tumor Cells

	IC_{50} in vitro (m*M*)		
Tumor of origin/cell line	Sulindac	Naproxen	Salicylate
Colorectal cancer			
HT115	0.5	1.5	7.5
DLD-1	0.5	2.5	6.0
HCT-15	0.4	1.5	6.0
Bladder cancer			
RT4	0.6	4.4	ND
T24	1.1	4.5	ND
RT112	0.8	4.2	ND
Prostate cancer			
PC3	0.3	5.5	ND
Ovarian cancer			
A2780	0.1	1.4	ND
Breast cancer			
MCF-7	0.7	4	ND

ND, no data.

cycle *(48)*. NSAIDs are also known to generate ROS, which can induce SSAT again, linking NSAIDs and the polyamine pathway to cell death and apoptosis.

One of the strong arguments against COX inhibition being a major factor in chemoprevention was that the products of the enzyme reaction, the PGs, could not reverse the growth inhibitory effects. On the other hand, when polyamines were added back to NSAID-inhibited cells, it was found that both in bladder and colorectal cancer cells that polyamine content was increased, apoptosis and cell number showed partial recovery *(32)*, supporting the case for polyamine involvement in the antiproliferative effect. We cannot rule out the possibility that the addition of polyamines *per se* may prevent apoptosis. For example, there is evidence that exogenous polyamines, particularly

spermine, can prevent activation of caspase 3 and apoptosis *(49)*. A more detailed analysis of the timing of each response would shed more light on whether polyamine depletion is cause or consequence of NSAID-induced cell death.

Another interesting development is the discovery that polyamines negatively regulate COX-2 expression in CaCo-2 cells probably through posttranscriptional mechanism involving eIF-5A *(50)*. This opens up the possibility of a more coordinated regulation of tumor prevention by both COX and polyamines. These observations are limited currently to colon and bladder cancer cells, but the hypothesis that polyamine-dependent and COX-independent mechanisms of toxicity occur in normal gut lining cells is under investigation. It does appear from initial in vitro observations that NSAID toxicity is an order of magnitude more pronounced in colon cancer cells than normal colonic cells (Wallace et al., unpublished observations). This would seem to indicate that polyamine-dependent mechanisms of toxicity in tumor cells may be dependent on the increased ODC activity, which is required for the initial events of apoptosis and the high polyamine content ubiquitously found in tumor cells.

If we accept that the polyamine pathway is part of the signaling cascade by which NSAIDs inhibit the growth of tumor cells then logically inhibitors of polyamine biosynthesis may be potentially useful chemopreventative agents. Several chemoprevention trials are currently underway using DFMO alone and in combination with NSAIDs and initial results seem promising *(51)*.

6. Summary

NSAIDs are an important class of drugs available both over the counter and by prescription. They have a range of therapeutic benefits not the least of which are only now being exploited: namely, their chemopreventive effects. On balance, the evidence supports a role for NSAIDs in cancer prevention with, in our opinion, colorectal cancer just being the first of several cancers to show a response. The mechanism of the chemopreventive effects is much less clear cut than the analgesic, antipyretic, and anti-inflammatory actions. The latter are mediated unequivocally via inhibition of COX enzymes. The former may involve COX inhibition but as a secondary effect. Our data suggest a role for the polyamines and their metabolic pathways as mediators of the NSAID-induced cytotoxicity through both polyamine depletion sensitizing cells to apoptosis and the oxidation of the polyamines *per se* generating ROS to further induce cell death. We await with interest further developments in this exciting area.

Although polyamine depletion may be beneficial in the prevention of tumor development, it appears that it may have negative effects on effective restitution after NSAID injury. It remains to be seen how these two factors can be resolved, but it has not been demonstrated conclusively that polyamine depletion by NSAIDs causes toxicity in normal GI epithelial cells. Our laboratory has demonstrated that the NSAIDs are more toxic to tumor cells in vitro than normal untransformed cells. Therefore, polyamine depletion may potentiate, not cause, NSAID-induced GI toxicity and prevent the healing process in normal epithelial cells, whereas in tumor cells, where polyamines are required both for transformation and growth, polyamine depletion may be directly responsible for the toxicity and apoptosis induced by the NSAIDs. In either case, there

is a substantial volume of work that suggests the polyamines play a protective role in GI physiology in response to injury, as well as offering a viable pathway for the targeting of chemopreventive agents.

References

1. Vane, J. R., Flower, R. J., and Botting, R. M. (1990) History of aspirin and its mechanism of action. *Stroke* **21,** IV12–IV23.
2. Vane, J. R. (1971) Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat. New Biol.* **231,** 232–235.
3. Ricchi, P., Zarrilli, R., Di Palma, A., and Acquaviva, A. M. (2003) Nonsteroidal anti-inflammatory drugs in colorectal cancer: From prevention to therapy. *Br. J. Cancer* **88,** 803–807.
4. Morita, I., Schindler, M., Regier, M. K., et al. (1995) Different intracellular locations for prostaglandin endoperoxide H synthase-1 and -2. *J. Biol. Chem.* **270,** 10,902–10,908.
5. Smith, W. L. and DeWitt, D. L. (1995) Biochemistry of prostaglandin endoperoxide H synthase-1 and synthase-2 and their differential susceptibility to nonsteroidal anti-inflammatory drugs. *Semin. Nephrol.* **15,** 179–194.
6. Charalambous, M. P., Maihofner, C., Bhambra, U., Lightfoot, T., and Gooderham, N. J. Colorectal Cancer Study Group. (2003) Upregulation of cyclooxygenase-2 is accompanied by increased expression of nuclear factor-kappa B and I kappa B kinase-alpha in human colorectal cancer epithelial cells. *Br. J. Cancer.* **88,** 1598–1604.
7. Wolff, H., Saukkonen, K., Anttila, S., Karjalainen, A., Vainio, H., and Ristimaki, A. (1998) Expression of cyclooxygenase-2 in human lung carcinoma. *Cancer Res.* **58,** 4997–5001.
8. Tucker, O. N., Dannenberg, A. J., Yang, E. K., et al. (1999) Cyclooxygenase-2 expression is up-regulated in human pancreatic cancer. *Cancer Res.* **59,** 987–990.
9. Shiff, S. J. and Rigas, B. (1999) The role of cyclooxygenase inhibition in the antineoplastic effects of nonsteroidal antiinflammatory drugs (NSAIDs). *J. Exp. Med.* **190,** 445–450.
10. Chan, T. A. (2002) Nonsteroidal anti-inflammatory drugs, apoptosis, and colon-cancer chemoprevention. *Lancet Oncol.* **3,** 166–174.
11. McCarthy, D. M. (1999) Comparative toxicity of nonsteroidal anti-inflammatory drugs. *Am. J. Med.* **107,** 37S–46S.
12. Lanza, F. L., Rack, M. F., Simon, T. J., et al. (1999) Specific inhibition of cyclooxygenase-2 with MK-0966 is associated with less gastroduodenal damage than either aspirin or ibuprofen. *Aliment Pharmacol. Ther.* **13,** 761–767.
13. Hawkey, C. J. and Langman, M. J. (2003) Non-steroidal anti-inflammatory drugs: Overall risks and management. Complementary roles for COX-2 inhibitors and proton pump inhibitors. *Gut* **52,** 600–608.
14. Cronstein, B. N. and Weissmann, G. (1995) Targets for antiinflammatory drugs. *Annu. Rev. Pharmacol. Toxicol.* **35,** 449–462.
15. Gierse, J. K., McDonald, J. J., Hauser, S. D., Rangwala, S. H., Koboldt, C. M., and Seibert, K. (1996) A single amino acid difference between cyclooxygenase-1 (COX-1) and -2 (COX-2) reverses the selectivity of COX-2 specific inhibitors. *J. Biol. Chem.* **271,** 15,810–15,814.
16. Cryer, B. and Dubois, A. (1998) The advent of highly selective inhibitors of cyclooxygenase—a review. *Prostaglandins Other Lipid Mediat.* **56,** 341–361.
17. Silverstein, F. E., Faich, G., Goldstein, J. L, et al. (2000) Gastrointestinal toxicity with celecoxib vs nonsteroidal anti-inflammatory drugs for osteoarthritis and rheumatoid arthritis: The CLASS study: A randomized controlled trial. Celecoxib Long-term Arthritis Safety Study. *JAMA* **284,** 1247–1255.

18. del Soldato, P., Sorrentino, R., and Pinto, A. (1999) NO-aspirins: A class of new anti-inflammatory and antithrombotic agents. *Trends Pharmacol. Sci.* **20,** 319–23.
19. Bjorkman, D. J. (1998) The effect of aspirin and nonsteroidal anti-inflammatory drugs on prostaglandins. *Am. J. Med.* **105,** 8S–12S.
20. Wallace, J. L., McKnight, W., Reuter, B. K., and Vergnolle, N. (2000) NSAID-induced gastric damage in rats: requirement for inhibition of both cyclooxygenase 1 and 2. *Gastroenterology* **119,** 706–714.
21. Warner, T. D., Giuliano, F., Vojnovic, I., Bukasa, A., Mitchell, J. A., and Vane, J. R. (1999) Nonsteroid drug selectivities for cyclo-oxygenase-1 rather than cyclo-oxygenase-2 are associated with human gastrointestinal toxicity: a full in vitro analysis. *Proc. Natl. Acad. Sci. USA* **96,** 7563–7568. Erratum in: *Proc. Natl. Acad. Sci. USA* (1999) **96,** 9666.
22. Robert, A., Nezamis, J. E., Lancaster, C., and Hanchar, A. J. (1979) Cytoprotection by prostaglandins in rats. Prevention of gastric necrosis produced by alcohol, HCl, NaOH, hypertonic NaCl, and thermal injury. *Gastroenterol.* **77,** 433–443.
23. Fang, W. F., Broughton, A., and Jacobson, E. D. (1977) Indomethacin-induced intestinal inflammation. *Am. J. Dig. Dis.* **22,** 749–760.
24. Wallace, J. L. (2000) How do NSAIDs cause ulcer disease? *Baillieres Best Pract. Res. Clin. Gastroenterol.* **14,** 147–159.
25. Nusrat, A., Delp, C., and Madara, J. L. (1992) Intestinal epithelial restitution. Characterization of a cell culture model and mapping of cytoskeletal elements in migrating cells. *J. Clin. Invest.* **89,** 1501–1511.
26. Szabo, I. L., Pai, R., Jones, M. K., Ehring, G. R., Kawanaka, H., and Tarnawski, A. S. (2002) Indomethacin delays gastric restitution: association with the inhibition of focal adhesion kinase and tensin phosphorylation and reduced actin stress fibers. *Exp. Biol. Med. (Maywood)* **227,** 412–424.
27. Wang, J. Y. and Johnson, L. R. (1990) Luminal polyamines stimulate repair of gastric mucosal stress ulcers. *Am. J. Physiol.* **259,** G584–G592.
28. Wang, J. Y., Wang, J., Golovina, V. A., Li, L., Platoshyn, O., and Yuan, J. X. (2000) Role of K(+) channel expression in polyamine-dependent intestinal epithelial cell migration. *Am. J. Physiol. Cell Physiol.* **278,** C303–C314.
29. Rao, J. N., Li, L., Golovina, V. A., et al. (2001) Ca2+-RhoA signaling pathway required for polyamine-dependent intestinal epithelial cell migration. *Am. J. Physiol. Cell Physiol.* **280,** C993–C1007.
30. Luk, G. D., Marton, L. J., and Baylin, S. B. (1980) Ornithine decarboxylase is important in intestinal mucosal maturation and recovery from injury in rats. *Science* **210,** 195–198.
31. Turchanowa, L., Dauletbaev, N,, Milovic, V., and Stein, J. (2001) Nonsteroidal anti-inflammatory drugs stimulate spermidine/spermine acetyltransferase and deplete polyamine content in colon cancer cells. *Eur. J. Clin. Invest.* **31,** 887–893.
32. Hughes, A., Smith, N. I., and Wallace, H. M. (2003) Polyamines reverse non-steroidal anti-inflammatory drug-induced toxicity in human colorectal cancer cells. *Biochem. J.* **374,** 481–488.
33. Babbar, N., Ignatenko, N. A., Casero, R. A. Jr., and Gerner, E. W. (2003) Cyclooxygenase-independent induction of apoptosis by sulindac sulfone is mediated by polyamines in colon cancer. *J. Biol. Chem.* **278,** 47,762–47,775.
34. Wang, J. Y. and Johnson, L. R. (1991) Polyamines and ornithine decarboxylase during repair of duodenal mucosa after stress in rats. *Gastroenterol.* **100,** 333–343.
35. Rao, J. N., Platoshyn, O., Li, L., et al. (2002) Activation of K(+) channels and increased migration of differentiated intestinal epithelial cells after wounding. *Am. J. Physiol. Cell Physiol.* **282,** C885–C898.

36. Guo, X., Rao, J. N., Liu, L., Rizvi, M., Turner, D. J., and Wang, J. Y. (2002) Polyamines regulate beta-catenin tyrosine phosphorylation via Ca(2+) during intestinal epithelial cell migration. *Am. J. Physiol. Cell Physiol.* **283,** C722–C734.
37. Banan, A., McCormack, S. A., and Johnson, L. R. (1998) Polyamines are required for microtubule formation during gastric mucosal healing. *Am. J. Physiol.* **274,** G879–G885.
38. Rao, J. N., Guo, X., Liu, L., et al. (2003) Polyamines regulate Rho-kinase and myosin phosphorylation during intestinal epithelial restitution. *Am. J. Physiol. Cell Physiol.* **284,** C848–C859.
39. Ray, R. M., Viar, M. J., McCormack, S. A., and Johnson, L. R. (2001) Focal adhesion kinase signaling is decreased in polyamine-depleted IEC-6 cells. *Am J. Physiol. Cell Physiol.* **281,** C475–C485.
40. Kaminska, B., Kaczmarek, L., and Grzelakowska-Sztabert, B. (1992) Inhibitors of polyamine biosynthesis affect the expression of genes encoding cytoskeletal proteins. *FEBS Lett.* **304,** 198–200.
41. Rao, J. N., Li, L., Bass, B. L., and Wang, J. Y. (2000) Expression of the TGF-beta receptor gene and sensitivity to growth inhibition following polyamine depletion. *Am. J. Physiol. Cell Physiol.* **279,** C1034–C1044.
42. Wong, S., Appleberg, M., Ward, C. M., and Lewis, D. R. (2004) Aspirin resistance in cardiovascular disease: A review. *Eur J. Vasc. Endovasc. Surg.* **27,** 456–465.
43. Giardiello, F. M., Offerhaus, G. J., and DuBois, R. N. (1995) The role of nonsteroidal anti-inflammatory drugs in colorectal cancer prevention. *Eur. J. Cancer* **31A,** 1071–1076.
44. Sandler, R. S., Galanko, J. C., Murray, S. C., Helm, J. F., and Woosley, J. T. (1998) Aspirin and nonsteroidal anti-inflammatory agents and risk for colorectal adenomas. *Gastroenterol.* **114,** 441–447.
45. Hanif, R., Pittas, A., Feng, Y., et al. (1996) Effects of nonsteroidal anti-inflammatory drugs on proliferation and on induction of apoptosis in colon cancer cells by a prostaglandin-independent pathway. *Biochem. Pharmacol.* **52,** 237–245.
46. Lindsay, G. S. and Wallace, H. M. (1999) Changes in polyamine catabolism in HL-60 human promyelogenous leukaemic cells in response to etoposide-induced apoptosis. *Biochem. J.* **337,** 83–87.
47. Parchment, R. E. and Pierce, G. B. (1989) Polyamine oxidation, programmed cell death, and regulation of melanoma in the murine embryonic limb. *Cancer Res.* **49,** 6680–6686.
48. Wallace, H. M., Fraser, A. V., and Hughes, A. (2003) A perspective of polyamine metabolism. *Biochem. J.* **376,** 1–14.
49. Moffatt J, Hashimoto M, Kojima A, et al. (2000) Apoptosis induced by 1′-acetoxychavicol acetate in 49. Ehrlich ascites tumor cells is associated with modulation of polyamine metabolism and caspase-3 activation. *Carcinogenesis* **21,** 2151–2157.
50. Parker, M. T. and Gerner, E. W. (2002) Polyamine-mediated post-transcriptional regulation of COX-2. *Biochimie* **84,** 815–819.
51. Turini, M. E. and DuBois, R. N. (2002) Primary prevention: phytoprevention and chemoprevention of colorectal cancer. *Hematol. Oncol. Clin. North Am.* **16,** 811–840

16

Role of Polyamines in the Control of the Immune Response in the Brain

Denis Soulet and Serge Rivest

1. Introduction

Polyamines are essential polycations involved in a large variety of biological functions, including modulation of the nucleic acid conformation, RNA export and their degradation, protein synthesis, eIF-5A posttranslational modification, signal transduction, cell growth and differentiation, and tumor progression *(1–3)*. These various aspects in the biology of polyamines are addressed in other sections of this book.

In the nervous system, one of the known functions of polyamines is their ability to interact with ion channels and to control membrane potential and excitability of particular groups of cells *(4–6)*. They are involved in the activation, inhibition, and blockage of *N*-methyl-D-asparate (NMDA) receptors *(7–9)*. Polyamines are also able to block specific subtypes of calcium-permeant kainate and a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors *(10)*. Furthermore, they are able to block Kir channels *(11–13)*, GMPc-dependent channels *(14)*, acetylcholine receptor channels *(15,16)*, and calcium and sodium membrane potential-dependant channels *(17,18)*.

Overactivation of the excitatory amino acid (EAA) receptors (NMDA, kainate, AMPA receptors) by polyamines can lead to neuronal death via an excitotoxicity type of phenomenon. Glutamate-induced excitotoxicity is involved in central nervous system (CNS) damages and chronic neurodegenerative diseases *(19,20)*. The prolonged calcium influx leads to a depolarization of the mitochondrial organelles *(21,22)*, which is followed by the decoupling of the electron transport with the adenosine triphosphate production *(23,24)*. Then, the release of free radicals initiate the excitotoxic damages *(25)* that can accumulate during long periods and contribute to the apparition of neuronal atrophy and demyelinating episodes. This process may be at the origin of various neurodegenerative diseases, such as Alzheimer's disease and amyotrophic lateral sclerosis (ALS). Polyamines have also been found to play a critical role in the neuronal damage during and after cerebral ischemia *(26)*.

As indicated, polyamines are involved in neurodegenerative processes *(27)*, but they can also protect neurons from cell death after axotomy *(28)*. On the other hand,

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increase in polyamine metabolism was found to be neurotoxic via glutamate-induced excitotoxic events and oxidative byproducts originating from the polyamine interconversion pathway *(29)*. In contrast, this pathway has been shown to play a critical role in the neuronal survival after cerebral ischemia *(30)*. Spermine is also able to act as a free radical scavenger *(31)*, which could be a neuroprotective mechanism in the presence of free radicals in the cerebral environment. It is also possible that ornithine decarboxylase (ODC) contributes to the growth and plasticity of specific groups of neurons because polyamines are involved in the development of the CNS *(32)* and axonal regeneration after injury *(33)*. Spermine is produced at high levels in regenerating tissues and is released into the extracellular medium during cellular lysis *(34)*. Such high levels of polyamines have previously been reported to be toxic for the brain, especially when oxidative products are generated by the interconversion pathway (extensively reviewed in ref. *35*). Through the literature, one can appreciate the ambivalent role of polyamines *(32)*, which obviously depends on several variables. This chapter summarizes the role of these small molecules in the control of immune proteins and whether such an interaction is beneficial or detrimental for the brain cells.

2. Polyamines in Innate Immune Response In Vitro: Modulation of Cytokine Release and Nitric Oxide Biosynthesis

The innate immune system is characterized by an unspecific and rapid response to microbial components as various as peptidoglycan, lipoproteins, lipoteichoic acid, bacterial CpG DNA, and the endotoxin lipopolysaccharide (LPS) *(36,37)*. The latter is a major glycolipid constituting the outer membrane of Gram-negative bacteria. LPS is well known to activate macrophages and trigger the release of proinflammatory cytokines (Fig. 1) and therefore is widely used as a model of the innate immune system *(38)*. Acute endotoxemia provokes a sharp and transient induction of proinflammatory signaling events and transcription of genes that encode cytokines, chemokines, enzymes, and proteins of the complement system, and Toll-like receptor 2 (TLR2) in the CNS (for a review, *see* ref. *39*). Polyamines have recently been found to alter the inflammatory response in vitro *(40,41)*. They act as negative immune regulators on lymphocytes *(42)*, neutrophil locomotion *(43)*, and natural killer (NK) cell activity *(44)*. The endotoxin LPS is able to increase ODC messenger RNA (mRNA) expression in monocytes *(45)* and spermine inhibits expression of the inducible isoform of nitric oxide (NO) synthase (iNOS) in LPS-treated J774.2 macrophages *(46)*. The work by Molitor and colleagues has shown that LPS can induce ODC activity in mouse B lymphocytes *(47)*. Intracellular polyamine levels were also found to alter macrophage-mediated cytotoxicity in vitro *(48)*. Furthermore, the release of proinflammatory cytokines by monocytes is inhibited by spermine through a spermine uptake-dependant mechanism *(49)*. Spermine is able to increase interleukin (IL)-10 synthesis and suppress IL-12p40 and interferon-g production in LPS-stimulated macrophages *(40)*, whereas ODC activity is enhanced in the lungs, liver, and spleen after LPS exposure *(50)*. These data indicate that, at least in vitro, spermine has anti-inflammatory properties (reviewed in ref. *51*). Thus, a narrow link seems to exist between the inflammatory reaction and polyamines. These effects of polyamines on cytokine production in vitro are summarized in the Table 1.

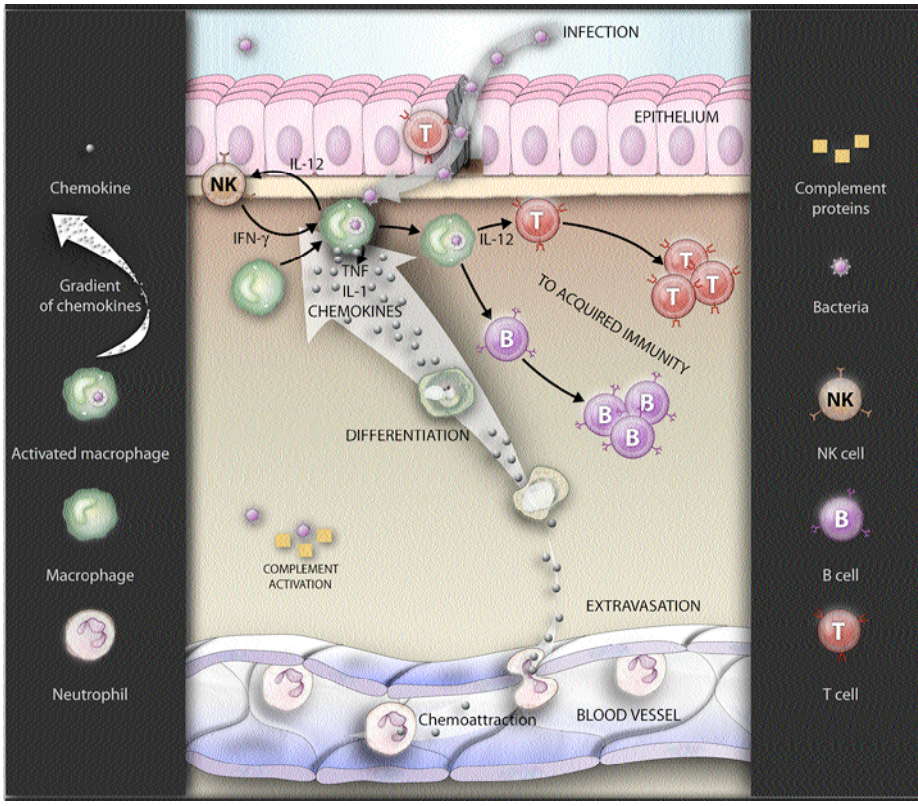

Fig. 1. Mediators of innate immunity and polyamines. This figure depicts a central role of macrophages in the innate immune response and the potential involvement of polyamines in these events. Macrophages are first activated by pathogen components, called pathogen-associated molecular patterns. Then myeloid cells release proinflammatory cytokines (TNF-a, interleukin 1, interleukin 12), which can activate natural killer (NK) cells. NK cells release the cytokine interferon-g (IFN-g), which further stimulates macrophages. A chemoattractant gradient takes place in response to the chemokine production by macrophages and endothelial cells. Neutrophils and monocytes are attracted from the lumen of the blood vessels to the parenchyma where the area of infection is located. Once into the parenchyma, monocytes differentiate into macrophages. Activated macrophages induce the adaptive immunity via the stimulation of B- and T-cells. This transfer from innate to adaptive immunity is highly dependent on interleukin-12. Macrophages play, therefore, a critical role in the control of the innate immune response and polyamines have a major impact on these events because of their ability to modulate the release of various proinflammatory molecules.

Another interesting point is the link between polyamine, immunity, and NO synthesis. iNOS has been initially described as an enzyme expressed in activated macrophages that generates NO from L-arginine. NO takes part in the elimination of intracellular microbial pathogens. In addition to its antibacterial role, NO controls the function of

Table 1
Effects of Polyamines in the Control of Cytokine Production In Vitro

		Effects				
Cytokine	Produced by	B-cells	T-cells	Macrophages	Hematopoietic cells	Other somatic cells
Interleukin-10	Lymphocytes (Th-2)	Increase MHC II	Inhibit Th-1	Inhibit cytokine release	Costimulate mastocyte growth	—
Interleukin-12	Macrophages, dendritic cells	—	Differentiation	—	—	—
Interferon-g	Lymphocytes (Th-1, CTL)	Differentiation IgG synthesis	Death	**Activation INCREASE MHC I and II**	NK cell activation	Antiviral, increase MHC I and II
TNF-a	Th-1, Th-2, CTL	—	—	**NO activation, induction and production**	—	—

The major effects are indicated in bold. Th-1, lymphocytes T CD4 auxiliary; Th-2, lymphocytes T CD4 inflammation; CTL, lymphocyte T CD8 cytotoxic; MHC, major histocompatibility complex.

NK cells and the expression of cytokines, such as the interferon-g and the transforming growth factor-b. It has recently been suggested that polyamines could inhibit the synthesis of NO because the metabolisms of polyamines and NO are partly interconnected via a common precursor, L-arginine (Fig. 2) *(52,53)*. Furthermore, NO could inhibit polyamine biosynthesis through the nitrosylation of the active ODC site *(54,55)*. Finally, spermine was found to inhibit iNOS expression in LPS-treated macrophages J774.2 *(53)*.

Taken together, many observations support the notion that polyamines are anti-inflammatory molecules. However, numerous studies were in a cell culture environment, which contains bovine serum and, thus, artificially high concentration of polyamine oxidase. This enzyme is well known to produce toxic byproducts of polyamines that could be at the origin of the anti-inflammatory effects of polyamines. Given the detrimental role of polyamines in excitotoxicity models in vivo and its potential involvement in neurodegenerative diseases, the following section presents the role of polyamines in the brain in vivo.

3. Modulation of the Innate Immune Response by Polyamines *In Vivo*

3.1. Increased Expression of ODC mRNA in the CNS of LPS-Treated Mice Vs Control

A model of systemic endotoxemia was used for its ability to increase the innate immune response in the brain, which is associated with transcriptional activation of numerous proinflammatory genes in microglial cells *(39,56–59)*. Our distribution of ODC mRNA in vehicle-treated animals was in accordance with a previous report *(60)*. However, the hybridization signal was clearly more intense in the brain of mice that received a single systemic bolus of LPS *(61)*. The medulla was particularly sensitive to the treatment and exhibited very strong expression levels, especially at 24 h after LPS administration. The data that a single systemic bolus of LPS caused a robust increase in ODC mRNA expression in numerous structures across the brain suggested a potential role of polyamines in the neuroinflammatory cascade of events *(61)*.

3.2. ODC Activity in the CNS of Vehicle- and LPS-Treated Mice

The ODC protein is highly regulated at the levels of transcription and translation, as well as at the posttranslational level. Furthermore, ODC enzyme has a very short half-life. To ascertain that LPS-induced increase in *ODC* gene expression was really associated with putrescine biosynthesis, ODC activity was measured in control mice and in mice that were treated with a suicide inhibitor of ODC, the difluoromethylornithine (DFMO). ODC activity was strongly induced by sixfold 3 h after a single systemic injection of LPS, which indicates that the latter is able to increase the biosynthesis of putrescine in the brain *(61)*. The enzymatic activity was reduced by 50% in mice that had access to DFMO in their drinking water for 2 d before the systemic LPS challenge. These data demonstrate the ability of DFMO to inhibit the effects of the LPS-induced ODC activity in the brain. Thus the potential that polyamines—or at least putrescine—are overproduced in the brain during endotoxemia exists.

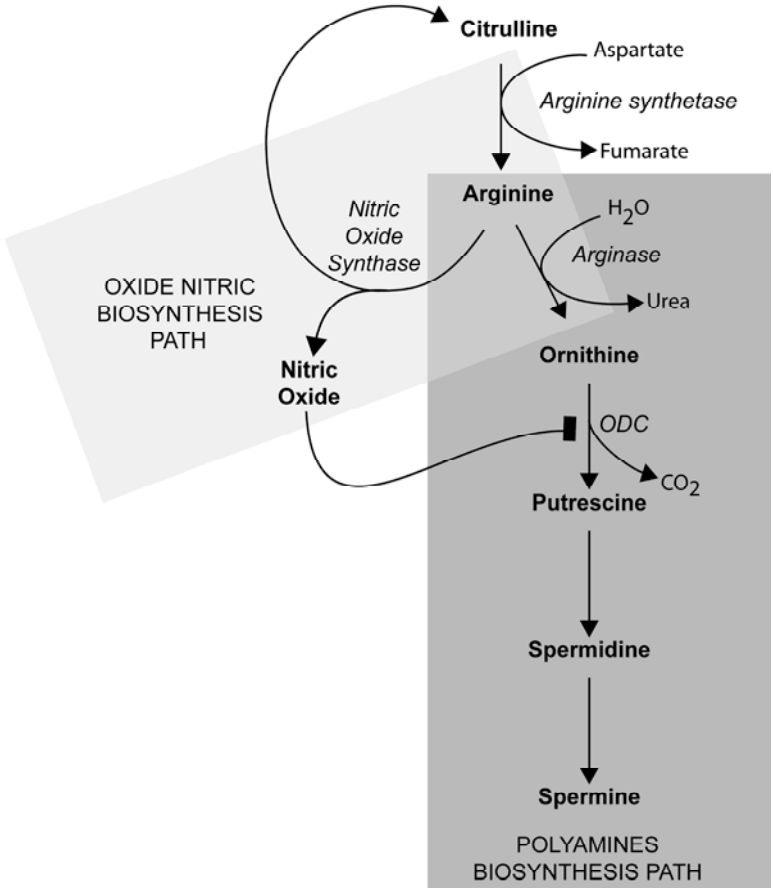

Fig. 2. Interrelationship between the polyamine and metabolic pathways of the nitric oxide. *See* Subheading 2 in text for details.

3.3. ODC Protein is Expressed in Neurons and Microglia Cells

To ensure the cellular localization of ODC in the untreated mouse brain, a multiple labeling approach was performed. Cytoplasmic ODC protein colocalized nicely with the specific marker of neuronal nuclei NeuN, which provided the evidence that neurons have the ability to produce polyamines *(61)*. ODC-immunoreactive cells also colocalized with a marker of microglial cells, iba1. Moreover, $ODC^+/NeuN^+$ cells were more numerous and widely distributed than $ODC^+/iba1^+$ cells across the cerebral tissue of mice injected systemically with the bacterial cell wall component. Microglial cells are the resident macrophages of the CNS and play a critical role in the innate immune response in the brain.

Of interest is that ODC is expressed in regions that are highly sensitive to excitotoxic insults. Although calcium permeant NMDA receptors are widely distributed in

the neurons of the CNS and are involved in neuronal excitotoxicity, glial cells (i.e., astrocytes, microglia, and oligodendrocytes) are also vulnerable to the prolonged activity of AMPA and kainate receptors. Such a process is generally weak in astrocytes, but it depends on the regions. Microglia are even less sensitive to excitotoxicity, because they express glutamate receptors only in particular conditions, such as the Alzheimer's disease or during postischemic conditions *(62,63)*. On the other hand, oligodendrocytes are extremely vulnerable to excitotoxicity *(64)* because of their high constitutive levels in kainate receptors *(65)*. In contrast to neurons, oligodendrocytes do not express calcium-binding proteins to limit the excitotoxic effects of this ion *(66)*. Therefore, the ability of LPS to induce polyamine release in the extracellular medium could be detrimental to neurons and oligodendrocytes—and microglial cells in particular conditions. However, while regulation of gene-encoding ODC takes place within neurons and microglia, proinflammatory transcripts are upregulated essentially within microglia when exposed to LPS.

3.4. Time-Related Inhibition of TLR2 mRNA by DFMO

TLR2 expression is transiently induced during acute endotoxemia and is largely used as a marker of proinflammatory events in the CNS *(67)*. Putrescine plays a critical role in this innate immune response, because TLR2 expression levels were much higher across the cerebral tissue of animals that had no free access to DFMO before the systemic LPS challenge *(61)*. Inhibition of ODC by DFMO largely abolished the spreading of TLR2-expressing cells across the cerebral tissue and leptomeninges no longer displayed positive signal in mice treated with LPS and DFMO at all the times evaluated in our recently published study *(61)*. Microglia are under the control of polyamines because DFMO significantly prevented the increase in TLR2 and cytokine mRNA levels in response to circulating LPS. That DFMO dramatically inhibited the effects of LPS on TLR2 expression in regions devoid of a blood–brain barrier indicates that decrease in putrescine synthetic capacity is a profound endogenous anti-inflammatory mechanism in the brain.

3.5. Induction of TLR2 and Tumor Necrosis Factor-a Transcripts by Intracerebroventricular Injection of Spermine

The effects of LPS on TLR2 and tumor necrosis factor-a gene expression can be exacerbated in the mouse brain treated with spermine. Animals that received an intracerebral spermine administration before the systemic LPS injection exhibited a profound transcriptional activation of TLR2 in the choroids plexus, circumventricular organs, leptomeninges, microcapillaries, and within small-scattered cells across the cerebral tissue *(61)*. Tumor necrosing factor-a-expressing cells were widely spread out throughout the CNS of mice that were killed 6 h after being injected with spermine before the endotoxin. Interestingly, polyamines have the ability to modulate the immune response only in presence of LPS, and spermine alone is unable of mimicking the effects of the endotoxin. In this model, polyamines are not direct ligands for activating proinflammatory signaling and gene expression by microglial cells. The brains of animals that received DFMO or spermine alone were comparable to those of vehicle-treated mice.

However, polyamines seem required to transduce the secondary signal taking place across microglial cells of LPS-treated mice.

3.6. Functional Role of Polyamines in a Model of Inflammation-Induced Neurotoxicity

To understand the functional role of polyamines in the CNS as being neurotoxic or neuroprotective molecules, we used a model of neurodegeneration caused by an exaggerated inflammatory response. Glucocorticoids are one of the most powerful endogenous suppressors for the innate immune response *(68)* and are essential molecules to avoid exaggerated responses during immunogenic challenges. As expected, the inflammatory response lasted longer in the brain of animals that received the glucocorticoid receptor antagonist RU486 before the intracerebral LPS infusion *(69)*. In this model, a single bolus of LPS caused a rapid and severe neurodegeneration only in RU486-pretreated animals *(70)*. Brain damages were detected with Nissl stain 3 d after the dual treatment combining intracerebral LPS and systemic RU486. Infusion of the endotoxin alone directly into the brain parenchyma failed to provoke neurodegeneration despite the transient innate immune reaction. Pretreatment with DFMO essentially abolished the effects of the combined injections of RU486 and LPS. In addition, DFMO was able to increase dramatically the survival rate of mice treated with RU486/LPS *(61)*. The decrease in putrescine levels by DFMO before the RU486/LPS cotreatment was able to increase the survival rate from 7 to 66% and to protect LPS-induced neurotoxicity because of the inhibition of glucocorticoid receptors in the brain. Polyamines are therefore powerful neurotoxic molecules in this model of exaggerated innate immune reaction.

3.7. How Do Polyamines Modulate the Innate Immune Response in the CNS?

The physiological relevance of such an interaction between polyamines and the innate immune system in the brain has yet to be clearly established. Our data that support polyamines acting as proinflammatory molecules are in disagreement with reports in which spermine in vitro was found to inhibit cytokine synthesis in macrophages in culture *(49,51,71)*. However, this discrepancy can be explain by the fact that our experiments were performed in an in vivo model in the brain where microglial cells, the CNS-resident macrophages, are in narrow paracrine relationships with other cells, such as astrocytes and neurons. It is also noteworthy that the brain possesses its own attributes, among them a distinct anatomy compared with the other organs in the body, with a particular vascular system formed by the blood–brain barrier and the unique presence of microglia cells as immune cells. Another major difference is that, unlike in vivo systems, LPS may not be eliminated in culture and chronic exposure to the endotoxin is known to cause tolerance of immune cells to this ligand *(72)*. Tolerant macrophages may behave quite differently to polyamines from parenchymal microglia that are activated in an acute manner by LPS or other pathogen-associated molecular patterns. Actually, several possibilities could be proposed to account for our observations (Fig. 3). Polyamines may directly alter LPS-induced signal transduction and gene expression in microglial cells, leading to an upregulation of the *TLR2* gene. Alternatively,

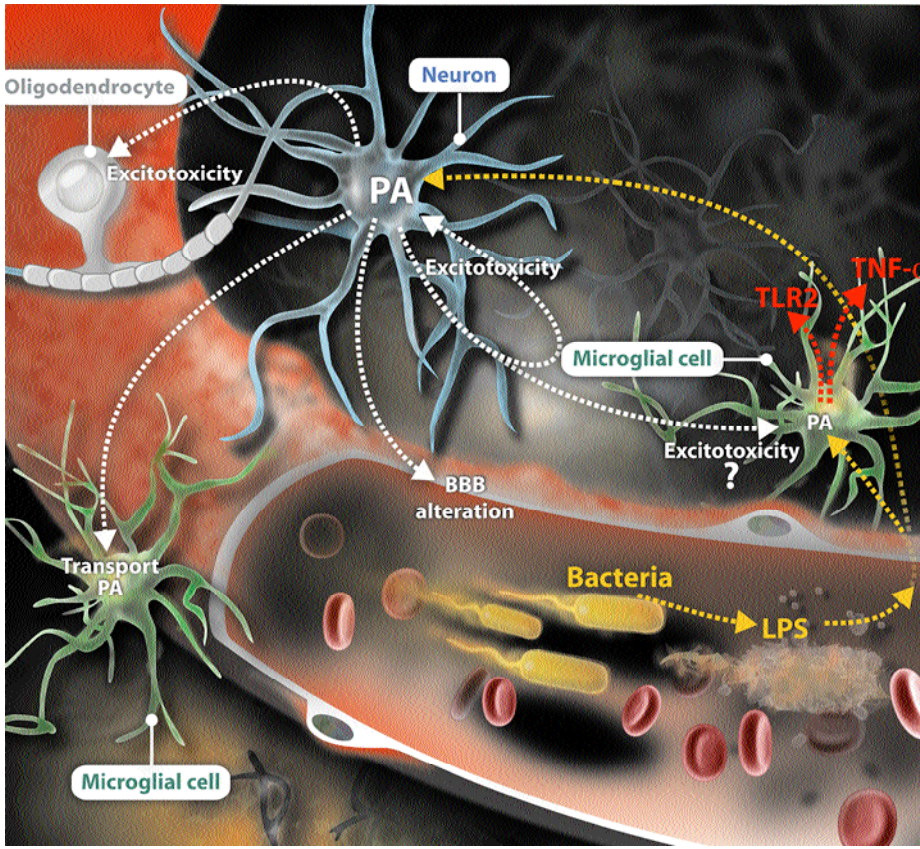

Fig. 3. Hypothetical model proposed for the role of polyamines in the control of the innate immune response in the brain. During endotoxemia, lipopolysaccharide is released into the blood flow and activates directly or indirectly ornithine decarboxylase (ODC) enzyme and therefore the biosynthesis of polyamines (PA) by microglial cells. This causes a robust increase in the expression levels of different immune transcripts, such as Toll-like receptor 2 (TLR2) and tumor necrosis factor (TNF)-a. The endotoxin has also the ability to stimulate ODC expression in neurons that would lead to a PA release in the extracellular milieu. PA could then be transported in microglia (via a polyamine transport system) and further activate *TLR2* and *TNF-a* gene transcription. Another possibility would be that free extracellular PA triggers excitotoxicity in neurons, oligodendrocytes, and possibly microglia. Finally, PA could open the blood–brain barrier and allow pathogen-associated molecular patterns and proinflammatory molecules to enter the brain, which may exacerbate the damages and the immune response. LPS, lipopolysaccharide.

spermine released from neurons and microglia could exert toxic effects by an excitotoxic process via over activation of NMDA/AMPA/kainate receptors, which could trigger an early innate immune reaction and gene expression by microglial cells. Although the concentration in polyamine oxidase in the brain is very low, the possibility that

polyamines are oxidized to produce toxic byproducts at the origin of these immune effects cannot be ruled out. Another possibility would be the ability of extracellular spermine to act directly on microglia via a polyamine transport system and modulate the transcription of early genes involved in the control of the innate immune system. Finally, another possibility would be that polyamines could damage/permeabilize the blood brain barrier, allowing the entry of proinflammatory molecules in the brain.

4. Impact of Polyamines in Neurodegenerative Diseases

As seen in Subheading 3, polyamines play a major role in the control of the cerebral innate immune response during microbial challenges. These data may have a major clinical impact and suggest DFMO as a potential therapeutic drug to restrain neurodegeneration in brain disorders associated with inflammation. Indeed, polyamine levels in the blood are higher in patients suffering of Alzheimer's disease or ALS than healthy people of same age *(73)*. Furthermore, the ALS-Therapy Development Foundation has published that polyamines levels are four times higher in superoxide dismutase 1 (SOD1) mutant mice mimicking ALS disease than in their wild-type counterparts. Moreover, the treatment of SOD1 mice with DFMO increases significantly the survival rate of these ALS mice. Polyamines could act at three different levels to cause motor neuron cell death: increase susceptibility to excitotoxic insults, exaggerate the inflammatory response to pathogens, or provoke an inappropriate microglial reaction to endogenous debris released by degenerating motor neurons. Polyamines—as chronic inflammatory modulators—could also create a pro-cancer environment favorable for survival of tumor cells and their growth.

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References

1. Tabor, C.W. and Tabor, H. (1984) Polyamines. *Annu. Rev. Biochem.* **53,** 749–790.
2. Pegg, A. E., Secrist, J.A., 3rd, and Madhubala, R. (1988) Properties of L1210 cells resistant to alpha-difluoromethylornithine. *Cancer Res.* **48,** 2678–2682.
3. Fozard, J. R., Part, M. L., Prakash, N. J., et al. (1980) L-Ornithine decarboxylase: An essential role in early mammalian embryogenesis. *Science* **208,** 505–508.
4. Bianchi, L., Roy, M. L., Taglialatela, M., Lundgren, D. W., Brown, A. M., and Ficker, E. (1996) Regulation by spermine of native inward rectifier K+ channels in RBL-1 cells. *J. Biol. Chem.* **271,** 6114–6121.
5. Johnson, T. D. (1996) Modulation of channel function by polyamines. *Trends Pharmacol. Sci.* **17,** 22–27.
6. Williams, K. (1997) Interactions of polyamines with ion channels. *Biochem. J.* **325,** 289–297.
7. Kashiwagi, K., Pahk, A. J., Masuko, T., Igarashi, K., and Williams, K. (1997) Block and modulation of N-methyl-D-aspartate receptors by polyamines and protons: Role of amino

acid residues in the transmembrane and pore-forming regions of NR1 and NR2 subunits. *Mol. Pharmacol.* **52,** 701–713.
8. Williams, K. (1997) Interactions of polyamines with ion channels. *Biochem. J.* **325,** 289–297.
9. Williams, K. (1997) Modulation and block of ion channels: a new biology of polyamines. *Cell Signal* **9,** 1–13.
10. Kamboj, S. K., Swanson, G. T., and Cull-Candy, S. G. (1995) Intracellular spermine confers rectification on rat calcium-permeable AMPA and kainate receptors. *J. Physiol.* **486,** 297–303.
11. Fakler, B., Brandle, U., Bond, C., et al. (1994) A structural determinant of differential sensitivity of cloned inward rectifier K+ channels to intracellular spermine. *FEBS Lett.* **356,** 199–203.
12. Ficker, E., Taglialatela, M., Wible, B. A., Henley, C. M., and Brown, A. M. (1994) Spermine and spermidine as gating molecules for inward rectifier K+ channels. *Science* **266,** 1068–1072.
13. Lopatin, A. N., Makhina, E. N., and Nichols, C. G. (1994) Potassium channel block by cytoplasmic polyamines as the mechanism of intrinsic rectification. *Nature* **372,** 366–369.
14. Lu, Z. and Ding, L. (1999) Blockade of a retinal cGMP-gated channel by polyamines. *J. Gen. Physiol.* **113,** 35–43.
15. Haghighi, A. P. and Cooper, E. (1998) Neuronal nicotinic acetylcholine receptors are blocked by intracellular spermine in a voltage-dependent manner. *J. Neurosci.* **18,** 4050–4062.
16. Haghighi, A. P. and Cooper, E. (2000) A molecular link between inward rectification and calcium permeability of neuronal nicotinic acetylcholine alpha3beta4 and alpha4beta2 receptors. *J. Neurosci.* **20,** 529–541.
17. Huang, C. J. and Moczydlowski, E. (2001) Cytoplasmic polyamines as permeant blockers and modulators of the voltage-gated sodium channel. *Biophys. J.* **80,** 1262–1279.
18. Scott, R. H., Sutton, K. G., and Dolphin, A. C. (1993) Interactions of polyamines with neuronal ion channels. *Trends Neurosci.* **16,** 153–160.
19. Choi, D. W. (1988) Glutamate neurotoxicity and diseases of the nervous system. *Neuron* **1,** 623–634.
20. Lipton, S. A. and Rosenberg, P. A. (1994) Excitatory amino acids as a final common pathway for neurologic disorders. *N. Engl. J. Med.* **330,** 613–622.
21. Schinder, A. F., Olson, E. C., Spitzer, N. C., and Montal, M. (1996) Mitochondrial dysfunction is a primary event in glutamate neurotoxicity. *J. Neurosci.* **16,** 6125–6133.
22. White, R. J. and Reynolds, I. J. (1996) Mitochondrial depolarization in glutamate-stimulated neurons: an early signal specific to excitotoxin exposure. *J. Neurosci.* **16,** 5688–5697.
23. Beatrice, M. C., Palmer, J. W., and Pfeiffer, D. R. (1980) The relationship between mitochondrial membrane permeability, membrane potential, and the retention of Ca2+ by mitochondria. *J. Biol. Chem.* **255,** 8663–8671.
24. Gunter, T. E. and Pfeiffer, D. R. (1990) Mechanisms by which mitochondria transport calcium. *Am. J. Physiol.* **258,** C755–C786.
25. Coyle, J. T. and Puttfarcken, P. (1993) Oxidative stress, glutamate, and neurodegenerative disorders. *Science* **262,** 689–695.
26. Kirby, B. P. and Shaw, G. G. (2004) The neuroprotective effects of N1-dansyl-spermine in the gerbil model of cerebral ischaemia. *Brain Res.* **1011,** 74–83.
27. Morrison, L. D., Cao, X. C., and Kish, S. J. (1998) Ornithine decarboxylase in human brain: Influence of aging, regional distribution, and Alzheimer's disease. *J. Neurochem.* **71,** 288–294.

28. Gilad, G. M. and Gilad, V. H. (1983) Polyamine biosynthesis is required for survival of sympathetic neurons after axonal injury. *Brain Res.* **273,** 191–194.
29. Porcella, A., Carter, C., Fage, D., et al. (1991) The effects of N-methyl-D-aspartate and kainate lesions of the rat striatum on striatal ornithine decarboxylase activity and polyamine levels. *Brain Res.* **549,** 205–212.
30. Gilad, G. M. and Gilad, V. H. (1988) Early polyamine treatment enhances survival of sympathetic neurons after postnatal axonal injury or immunosympathectomy. *Brain Res.* **466,** 175–181.
31. Ha, H. C., Sirisoma, N. S., Kuppusamy, P., Zweier, J. L., Woster, P. M., and Casero, R. A., Jr. (1998) The natural polyamine spermine functions directly as a free radical scavenger. *Proc. Natl. Acad. Sci. USA* **95,** 11,140–11,145.
32. Shimizu, H., Kakimoto, Y., and Sano, I. (1965) Changes in concentration of polyamines in the developing mouse brain. *Nature* **207,** 1196–1197.
33. Cai, D., Deng, K., Mellado, W., Lee, J., Ratan, R., and Filbin, M. (2002) Arginase I and polyamines act downstream from cyclic AMP in overcoming inhibition of axonal growth MAG and myelin in vitro. *Neuron* **35,** 711.
34. Clarke, J. R. and Tyms, A. S. (1991) Polyamine biosynthesis in cells infected with different clinical isolates of human cytomegalovirus. *J. Med. Virol.* **34,** 212–216.
35. Seiler, N. (2000) Oxidation of polyamines and brain injury. *Neurochem. Res.* **25,** 471–490.
36. Lee, S. J. and Lee, S. (2002) Toll-like receptors and inflammation in the CNS. *Curr. Drug Targets Inflamm. Allergy* **1,** 181–191.
37. Wright, S. D. (1999) Toll, a new piece in the puzzle of innate immunity. *J. Exp. Med.* **189,** 605–609.
38. Taveira da Silva, A. M., Kaulbach, H. C., Chuidian, F. S., Lambert, D. R., Suffredini, A. F., and Danner, R. L. (1993) Brief report: shock and multiple-organ dysfunction after self-administration of Salmonella endotoxin. *N. Engl. J. Med.* **328,** 1457–1460.
39. Nguyen, M. D., Julien, J. P. and Rivest, S. (2002) Innate immunity: the missing link in neuroprotection and neurodegeneration? *Nat. Rev. Neurosci.* **3,** 216–227.
40. Hasko, G., Kuhel, D. G., Marton, A., Nemeth, Z. H., Deitch, E. A., and Szabo, C. (2000) Spermine differentially regulates the production of interleukin-12 p40 and interleukin-10 and suppresses the release of the T helper 1 cytokine interferon-gamma. *Shock* **14,** 144–149.
41. Messina, L., Arcidiacono, A., Spampinato, G., et al. (1990) Accumulation of ornithine decarboxylase mRNA accompanies activation of human and mouse monocytes/macrophages. *FEBS Lett.* **268,** 32–34.
42. Byrd, W. J., Jacobs, D. M., and Amoss, M. S. (1977) Synthetic polyamines added to cultures containing bovine sera reversibly inhibit in vitro parameters of immunity. *Nature* **267,** 621–623.
43. Ferrante, A., Maxwell, G. M., Rencis, V. O., Allison, A. C., and Morgan, D. M. (1986) Inhibition of the respiratory burst of human neutrophils by the polyamine oxidase-polyamine system. *Int. J. Immunopharmacol.* **8,** 411–417.
44. Quemener, V., Blanchard, Y., Chamaillard, L., Havouis, R., Cipolla, B., and Moulinoux, J. P. (1994) Polyamine deprivation: A new tool in cancer treatment. *Anticancer Res.* **14,** 443–448.
45. Zheng, S. A., McElwain, C. M., and Taffet, S. M. (1991) Regulation of mouse ornithine decarboxylase gene expression in a macrophage-like cell line: synergistic induction by bacterial lipopolysaccharide and cAMP. *Biochem. Biophys. Res. Commun.* **175,** 48–54.
46. Szabo, C., Southan, G. J., Wood, E., Thiemermann, C., and Vane, J. R. (1994) Inhibition by spermine of the induction of nitric oxide synthase in J774.2 macrophages: Requirement of a serum factor. *Br. J. Pharmacol.* **112,** 355–356.

47. Molitor, J., Pace, W., Stunz, L., Vigna, J., Louie, S., and Ashman, R. (1994) Induction of ornithine decarboxylase activity in mouse B lymphocytes. *Int. Immunol.* **6,** 1777–1784.
48. Tjandrawinata, R. R., Hawel, 3rd, L., and Byus, C. V. (1994) Regulation of putrescine export in lipopolysaccharide or IFN-gamma- activated murine monocytic-leukemic RAW 264 cells. *J. Immunol.* **152,** 3039–3052.
49. Zhang, M., Borovikova, L. V., Wang, H., Metz, C., and Tracey, K. J. (1999) Spermine inhibition of monocyte activation and inflammation. *Mol. Med.* **5,** 595–605.
50. Sugimoto, H., Matsuzaki, Y., Yuasa, K., et al. (1992) Estradiol-17 beta modifies the induction of spermidine/spermine N1-acetyltransferase activity in the liver of lipopolysaccharide-treated mice. *Liver* **12,** 147–151.
51. Zhang, M., Wang, H., and Tracey, K. J. (2000) Regulation of macrophage activation and inflammation by spermine: A new chapter in an old story. *Crit. Care Med.* **28,** N60–N66.
52. Southan, G. J., Szabo, C., and Thiemermann, C. (1994) Inhibition of the induction of nitric oxide synthase by spermine is modulated by aldehyde dehydrogenase. *Biochem. Biophys. Res. Commun.* **203,** 1638–1644.
53. Szabo, C., Southan, G. J., Thiemermann, C., and Vane, J. R. (1994) The mechanism of the inhibitory effect of polyamines on the induction of nitric oxide synthase: role of aldehyde metabolites. *Br. J. Pharmacol.* **113,** 757–766.
54. Bauer, P. M., Buga, G. M., Fukuto, J. M., Pegg, A. E., and Ignarro, L. J. (2001) Nitric oxide inhibits ornithine decarboxylase via S-nitrosylation of cysteine 360 in the active site of the enzyme. *J. Biol. Chem.* **276,** 34,458–34,464.
55. Ignarro, L.J., Buga, G. M., Wei, L. H., Bauer, P. M., Wu, G., and del Soldato, P. (2001) Role of the arginine-nitric oxide pathway in the regulation of vascular smooth muscle cell proliferation. *Proc. Natl. Acad. Sci. USA* **98,** 4202–4208.
56. Nadeau, S. and Rivest, S. (2000) Role of microglial-derived tumor necrosis factor in mediating CD14 transcription and nuclear factor kappa B activity in the brain during endotoxemia. *J. Neurosci.* **20,** 3456–3468.
57. Nadeau, S. and Rivest, S. (2001) The complement system is an integrated part of the natural innate immune response in the brain. *FASEB J.* **15,** 1410–1412.
58. Laflamme, N., Soucy, G., and Rivest, S. (2001) Circulating cell wall components derived from gram-negative, not gram-positive, bacteria cause a profound induction of the gene-encoding Toll-like receptor 2 in the CNS. *J. Neurochem.* **79,** 648–657.
59. Laflamme, N. and Rivest, S. (2001) Toll-like receptor 4: the missing link of the cerebral innate immune response triggered by circulating gram-negative bacterial cell wall components. *FASEB J.* **15,** 155–163.
60. Kilpelainen, P., Rybnikova, E., Hietala, O., and Pelto-Huikko, M. (2000) Expression of ODC and its regulatory protein antizyme in the adult rat brain. *J. Neurosci. Res.* **62,** 675–685.
61. Soulet, D. and Rivest, S. (2003) Polyamines play a critical role in the control of the innate immune response in the mouse central nervous system. *J. Cell. Biol.* **162,** 257–268.
62. Gottlieb, M. and Matute, C. (1997) Expression of ionotropic glutamate receptor subunits in glial cells of the hippocampal CA1 area following transient forebrain ischemia. *J. Cereb. Blood Flow Metab.* **17,** 290–300.
63. Kingham, P. J., Cuzner, M. L., and Pocock, J. M. (1999) Apoptotic pathways mobilized in microglia and neurones as a consequence of chromogranin A-induced microglial activation. *J. Neurochem.* **73,** 538–547.
64. Oka, A., Belliveau, M. J., Rosenberg, P. A., and Volpe, J. J. (1993) Vulnerability of oligodendroglia to glutamate: pharmacology, mechanisms, and prevention. *J. Neurosci.* **13,** 1441–1453.

65. Sanchez-Gomez, M. V. and Matute, C. (1999) AMPA and kainate receptors each mediate excitotoxicity in oligodendroglial cultures. Neurobiol. Dis. **6,** 475–485.
66. Baimbridge, K. G., Celio, M. R., and Rogers, J. H. (1992) Calcium-binding proteins in the nervous system. *Trends Neurosci.* **15,** 303–308.
67. Nguyen, M. D., Julien, J. P. and Rivest. S. (2002) Innate immunity: the missing link in neuroprotection and neurodegeneration? *Nat. Rev. Neurosci.* **3,** 216–227.
68. McKay, L. I. and Cidlowski, J. A. (1999) Molecular control of immune/inflammatory responses: Interactions between nuclear factor-kappa B and steroid receptor-signaling pathways. *Endocr. Rev.* **20,** 435–459.
69. Nadeau, S. and Rivest, S. (2002) Endotoxemia prevents the cerebral inflammatory wave induced by intraparenchymal lipopolysaccharide injection: Role of glucocorticoids and CD14. *J. Immunol.* **169,** 3370–3381.
70. Nadeau, S. and Rivest, S. (2003) Glucocorticoids play a fundamental role in potecting the brain during innate immune response. *J. Neurosci.* **23,** 5536–5544.
71. Zhang, M., Caragine, T., Wang, H., et al. (1997) Spermine inhibits proinflammatory cytokine synthesis in human mononuclear cells: A counterregulatory mechanism that restrains the immune response. *J. Exp. Med.* **185,** 1759–1768.
72. Nomura, F., Akashi, S., Sakao, Y., et al. (2000) Cutting edge: Endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface toll-like receptor 4 expression. *J. Immunol.* **164,** 3476–3479.
73. Gomes-Trolin, C., Nygren, I., Aquilonius, S. M., and Askmark, H. (2002) Increased red blood cell polyamines in ALS and Parkinson's disease. *Exp. Neurol.* **177,** 515–520.

17

Implication of Polyamines in Apoptosis of Immunoresponse Cells

Rupesh Chaturvedi and Keith T. Wilson

1. Introduction

In many diseases, including host response to pathogens, dysregulation of the immune system is a hallmark. An important component of such events is alterations in apoptosis of immune cells. This may include failure of apoptosis, but most commonly involves loss of immune function from death of immune cells. We will review the role of polyamines in immune cell apoptosis and provide specific insights based on our laboratory's work on the pathogenesis of *Helicobacter pylori* infection and the central role of polyamines in the associated apoptosis of macrophages.

2. General Overview of Immunity

2.1. Innate Immunity

As a framework for discussion, innate immunity generally refers to responses that do not require previous exposure to the immune stimulus. There have been tremendous advances in this field over the last decade, including the elucidation of the Toll-like receptors that are activated by recognition of pathogen-associated molecular patterns (for review, *see* ref. *1*). The innate immune system represents the "first line of defense" in the response to pathogens. Nonspecific activation by various stimuli from microorganisms or produced by the host can lead to important antimicrobial effects, but can also result in inflammation and injury resulting from release of inflammatory mediators such as cytokines, reactive oxygen species, and nitric oxide.

2.2. Adaptive Immunity

The adaptive immune response is considered the predetermined response to a previously identified immunological stimulus. Thus, the response is specific to a particular pathogen and involves immunological memory. However, the lines between adaptive and innate immunity are frequently blurred by the close interactions between pathways, such that stimulation of antigen-presenting cells, such as macrophages and dendritic

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cells, leads to activation and recruitment of lymphocytes and the development of T-helper cell-specific responses. As an example, in the case of *H. pylori* infection of the gastric mucosa, there is a resulting gastritis with chronic (lymphocytic) and active (neutrophilic) components that is driven by a variety of bacterial factors that stimulate epithelial cell and macrophage activation, as well as a T-helper 1 predominant lymphocyte response (reviewed in ref. *2*). Colonization of *H. pylori* can be abrogated by immunization of urease *(2)* indicating activation of the adaptive response, but urease is a major inducer of innate responses in monocytes and macrophages, such as cytokine and nitric oxide generation *(3)*.

2.3. Potential Role of Apoptosis in Immunity

This is a fascinating area of investigation, because apoptosis of immune cells has been linked to both beneficial and deleterious effects. When immune cells undergo apoptosis this can be a necessary event for the host to prevent propagation of inflammation. As an example, it has been argued that Crohn's disease, a chronic relapsing form of inflammatory bowel disease, results in part from a failure of activated T-helper cells to undergo apoptosis *(4)*. Similarly, apoptosis and consumption of infected cells by neutrophils may represent an important mechanism for elimination of pathogens. In contrast, apoptosis of infected cells has been hypothesized as a major cause for both proliferation of invasive organisms such as *Shigella*, and release of proinflammatory mediators *(5)*. Additionally, death of immune cells is potentially an important feature of the "immune escape" of pathogens and tumor cells.

3. Polyamines in Apoptosis

3.1. Introduction

Polyamines are important multifunctional cellular components and are required for cell growth and division. Polyamines also have been shown to be associated with cell death caused by apoptosis. The effects of the polyamines are not simple because they have been shown to cause both increases and decreases in apoptosis. These effects can be regulated by alterations in biosynthesis and catabolism, as discussed in Subheading 3.2. There is now substantial evidence that polyamines can cause either cell proliferation or cell death depending on the cell type and the nature of the stimuli to which the cell is exposed.

3.2. Effect of Polyamine Biosynthesis on Apoptosis

Ornithine decarboxylase (ODC) is one of the rate-limiting enzymes of polyamine biosynthesis. Cells have developed a complex regulatory mechanism, which precisely controls intracellular polyamine levels by the combined effects of *de novo* synthesis, back conversion, export, and uptake of polyamines. The exact role of polyamines in apoptotic pathways is still unclear. An elegant series of studies by Packham and Cleveland *(6)* linked increases in ODC activity to apoptosis in murine myeloid cells. Enforced expression of c-Myc caused increased ODC activity and apoptosis, both of which were inhibited by the ODC inhibitor, α-difluoromethylornithine (DFMO). Other studies have shown that inhibition of polyamine synthesis with DFMO

promotes resistance of intestinal epithelial cells (IECs) to apoptosis *(7)*. Specifically, depletion of polyamines by DFMO resulted in resistance to apoptotic cell death induced by the combination of tumor necrosis factor (TNF)-α and cycloheximide *(7)*, which is related to activation of nuclear factor-κB in IECs.

Apoptosis can occur as a consequence of mitochondrial membrane depolarization, which causes cytochrome c release from mitochondria to cytosol and activation of caspases *(8,9)*. This is associated with alterations in the levels and localization of Bcl-2 family proteins *(10)*. As such, polyamine depletion has been shown to decrease Bax translocation to mitochondria and inhibit cytochrome c release in camptothecin-treated rat IECs *(11)*.

In contrast, depletion of polyamines with sulindac sulfone in colon cancer cells induces apoptosis without stimulation *(12)*. Depletion of polyamines by various strategies has also has shown to increase apoptosis in cells such as head-and-neck squamous carcinoma cells *(13)*, human T-lymphoblastic leukemia cells *(14)*, and human gastric cancer cells *(15)*. Polyamine depletion also increases susceptibility of DFMO-treated rat IECs to staurosporine-induced apoptosis *(7)*.

There are differences between the polyamines in terms of their ability to induce apoptosis. Spermine analogs such as *bis*(ethyl)norspermine increase the sensitivity of some cells to apoptosis *(16)*. Recently, it has been shown that 5-fluorouracil and the spermine analog DENSPM synergistically increase tumor cell apoptosis in both p53 wild-type and p53-null variants *(17)*. However, 5-fluorouracil plus DFMO depleted putrescine, but did not produce synergistic cell killing, which was associated with loss of mitochondrial membrane potential, and release of cytochrome c *(17)*. The varying effects of DFMO on apoptosis may be attributed, in part, to the fact that it inhibits putrescine synthesis, but does not consistently deplete spermine levels and can even increase spermine accumulation *(18)*.

Alterations in polyamine homeostasis have been associated with induction of the mitochondrial-dependent apoptosis pathway. Opening of transition pores permits nonspecific bidirectional traffic of solutes across the inner mitochondrial membrane, leading to swelling of the organelle and release of cytochrome c and apoptosis-inducing proteins that activate the caspase cascade *(8)*. Depending on their cytosolic concentration, the metabolic status of the cell and the cell type, polyamines can inhibit *(19)* or induce mitochondrial-mediated apoptosis *(8,20)*. Although their inhibitory effect could reflect inhibition of the opening of the transition pores and retention of cytochrome c *(19)*, the inducing effect can be caused by generation of reactive oxygen species *(8,20)*, or the direct interaction of polyamines with the anionic phospholipids of the mitochondrial membrane *(8,20)*.

3.3. Effect of Polyamine Catabolism on Apoptosis

It was first postulated that H_2O_2 from extracellular serum amine oxidase-dependent catabolism of polyamines was a mediator of apoptosis in murine embryos, limb buds, and blastocytes *(21)*. The cytotoxic response of multiple tumor cell types to exposure to specific antitumor polyamine analogs has been attributed to the release of reactive oxygen species during polyamine catabolism *(22)*. Spermine and spermidine can be

metabolized by an oxidative process that results in the release of H_2O_2. In the originally described pathway of back conversion, there is a two-step process regulated by the rate-limiting enzyme spermidine/spermine N^1-acetyltransferase (SSAT) *(23,24)*. SSAT generates acetylspermine or acetylspermidine from spermine and spermidine, respectively, thus providing substrate for the peroxisomal polyamine oxidase, acetyl PAO (APAO). The latter produces spermidine and putrescine, respectively, and also generates the toxic byproducts H_2O_2 and 3-acetoaminopropanal that can initiate cell death *(24,25)*. Consistent with this, super-induction of SSAT by antitumor polyamine analogs has been implicated in the cytotoxic response of specific solid-tumor phenotypes to these agents *(22)*.

Intriguingly, when Niiranen et al. *(26)* generated mouse embryonic stem cells with targeted disruption of the *SSAT* gene, these cells exhibited a similar degree of polyamine depletion as the wild-type cells when exposed to polyamine analogs. Further, they showed that radiolabeled spermine was converted to spermidine in the mutant cell lines and actually this was more efficiently accomplished than in the parental cells. These results indicated that SSAT is not the only enzyme that can back convert spermine to spermidine *(26)*. Wang et al. *(27)* cloned an inducible form of polyamine oxidase, *PAOh1*, that was later named spermine oxidase (*SMO*) *(28)*, which directly and specifically oxidizes spermine to spermidine, generating H_2O_2 as a toxic byproduct *(27,28)*. Devereux et al. *(29)* have shown that polyamine analogs can induce SMO(PAOh1)-dependent apoptosis in human lung carcinoma cell lines.

3.4. Polyamines and Immune Cells and Apoptosis

Polyamine-associated apoptosis in immune cells is an emerging area of investigation. Ferioli et al. *(30)* have demonstrated that modulation of polyamine oxidase activity by glucocorticoid administration to rats correlated with the level of apoptosis in the thymus and spleen. In a subsequent study, this group showed that mitoguazone, which inhibits spermidine and spermine synthesis by blocking *S*-adenosylmethionine decarboxylase, protected thymocytes from both spontaneous and etoposide-induced apoptosis; this protective effect was associated with a decrease in polyamine oxidase activity and total polyamine levels *(31)*. In contrast, Jan et al. *(32)* reported that spontaneous and dexamethasone-induced apoptosis was inhibited by polyamines in rat thymocytes ex vivo.

In myelomonocytic HL-60 cells, etoposide-induced apoptosis correlated with polyamine efflux and decrease in polyamine content *(33)*. In contrast, apoptosis in these cells induced by 2-deoxy-D-ribose was prevented by treatment with DFMO, and exogenously added polyamines restored apoptosis *(34)*. The latter results were similar to those that we have observed in *H. pylori*-activated macrophages *(35)*, as discussed in Subheading 4. Similarly, in HL-60 cells, spermine has been reported to trigger cytochrome c release from mitochondria, and initiate activation of caspase-3, causing cell death via apoptosis *(36)*. These differences highlight the fact that the role of polyamines in apoptosis may vary depending on the nature of the death stimulus, as reported in IECs *(7)*.

There is also substantial evidence that polyamines can be antiapoptotic in lymphocytes. Nitta et al. *(37)* found that in the murine WEHI23 B-cell line, induction of

apoptosis by B-cell antigen receptor crosslinking was associated with decreased expression of polyamine biosynthetic enzymes, increased levels of polyamine catabolic enzymes, and decreased levels of intracellular polyamines. Addition of spermine repressed the apoptosis by inhibiting the loss of mitochondrial membrane depolarization *(37)*. This same group used inhibitors of polyamine biosynthesis to examine the effect of polyamine depletion and showed that decreased intracellular polyamine levels induced apoptotic cell death in WEHI231 cells *(38)*. Addition of exogenous polyamines reversed the observed features of apoptotic cell death. Similar effects were also observed in another human B-cell line, Ramos, and in a human T-cell line, Jurkat *(38)*.

Penning et al. (16) have used rat/mouse T-cell hybridoma-derived PC60 R55/R75 cells to study TNF-α-induced apoptosis. TNF-α caused a transient increase in ODC activity followed by a decrease and a gradual depletion of the polyamine levels. A reduction of the intracellular spermine levels with the polyamine synthesis inhibitors mitoguazone or CGP48644a, or with *bis*(ethyl)norspermine, potentiated the apoptosis caused by TNF-α, whereas addition of spermine reduced the apoptosis. Similar results were obtained in several other cell lines including CEM-CM3 human T-cells *(16)*.

In conclusion, the published data in immune cells clearly show both pro- and anti-apoptotic effects of polyamines. These results appear to vary depending on the cell type, the nature of the stimulus, and the specific polyamines that are manipulated in these systems. Additionally, the role of polyamine oxidation may be of paramount importance, as illustrated in Subheading 4.

4. *H. pylori*–Induced Apoptosis in Macrophages: A Disease-Specific Model

H. pylori is a Gram-negative, microaerophilic bacterium that selectively colonizes the human stomach. The current prevalence of infection is 30–40% in the United States and substantially higher in underdeveloped regions. *H. pylori* induces a vigorous mucosal immune response that fails to eradicate the organism, resulting in chronic gastritis that can lead to peptic ulcers, gastric adenocarcinoma, and gastric lymphoma. In addition to a chronic lymphocyte response, there is an activation of the innate immune response in monocytes and macrophages in particular, and recruitment of neutrophils. Our lab has focused on mechanisms whereby the innate immune response is dysregulated, and we have directly implicated polyamines in these events.

4.1. Importance of Arginase

We have demonstrated that *H. pylori* upregulates expression of inducible nitric oxide (NO) synthase (iNOS) in gastritis tissues in vivo *(39)* and in macrophages in vitro *(3)*, and iNOS-derived NO had been implicated as a causal factor in macrophage apoptosis in response to lipopolysaccharide plus interferon-γ *(40)*. When we identified that *H. pylori* also upregulated the arginase II isoform of arginase, which competes with iNOS for the same substrate, L-arginine, we determined the respective roles of arginase II and iNOS in *H. pylori*-induced macrophage apoptosis *(35)*. We were initially surprised to find that iNOS played no role because use of iNOS inhibitors or peritoneal macrophages from $iNOS^{-/-}$ mice had no effect on the apoptosis, whereas two different inhibitors of arginase effectively inhibited apoptosis *(35)*.

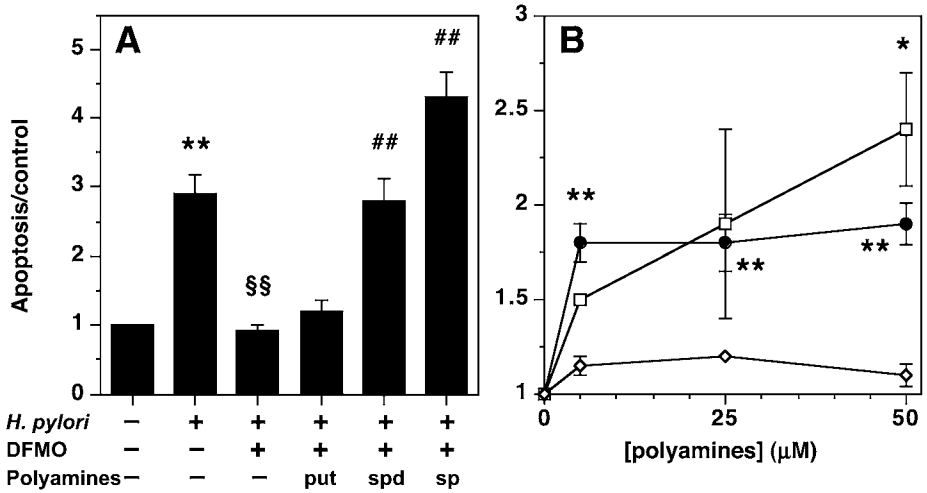

Fig. 1. ODC activity and polyamines enhance macrophage apoptosis. **(A)** *Helicobacter pylori*-stimulated macrophage apoptosis is ODC dependent. RAW 264.7 cells were cocultured with *H. pylori*, with or without DFMO (5 m*M*) and 5 μ*M* putrescine (put), spermidine (spd), or spermine (sp). After 24 h, macrophage apoptosis levels were determined by enzyme-linked immunosorbent assay. $^{**}p < 0.01$ vs control; §§$p < 0.01$ vs *H. pylori*-stimulated macrophages; ##$p < 0.01$ vs macrophages stimulated with *H. pylori* in the presence of DFMO. **(B)** Stimulation of macrophage apoptosis by polyamines alone. RAW 264.7 cells were treated for 24 h with different concentrations of putrescine, spermidine, or spermine. $^{*}p < 0.05$, $^{**}p < 0.01$ vs control macrophages. (Data shown are from ref. *35*, Copyright 2002, The American Association of Immunologists, Inc.)

4.2. Implication of Polyamines in Macrophage Apoptosis

In considering the likely mechanism of the latter effect, because arginase generates L-ornithine, and the latter is the substrate for ODC and the synthesis of polyamines, we determined if polyamines mediated the apoptosis. As shown in Fig. 1, inhibition of polyamine synthesis with DFMO completely abrogated the induction of apoptosis by *H. pylori (35)*. The apoptosis could be restored by add-back of spermidine or spermine, but not putrescine, and similarly addition of spermidine or spermine alone, but not putrescine, induced macrophage apoptosis. This led us to hypothesize that both arginase II and ODC induction was required for the *H. pylori*-induced macrophage apoptosis. Because macrophage apoptosis in response to a pathogen would be undesirable for the host, by leading to depletion of cells necessary for the innate immune response, we pursued additional studies to determine how polyamines could be causing the apoptosis.

4.3. Induction of ODC and SMO(PAO1) and the Relationship to Apoptosis

Because polyamine oxidation had been implicated in the apoptosis of other cell types, we hypothesized that *H. pylori* was capable of inducing this phenomenon in macrophages. Because *H. pylori* is a noninvasive organism in vivo, we established that

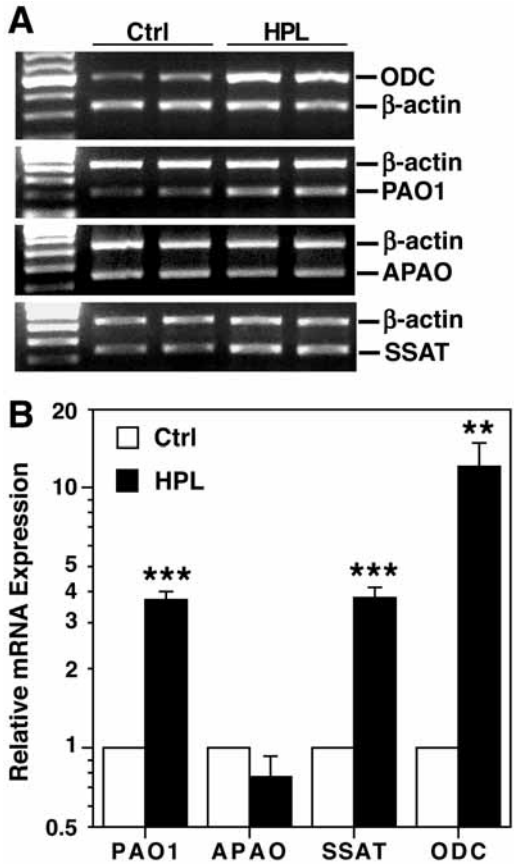

Fig. 2. *Helicobacter pylori* induces mRNA expression of ornithine decarboxylase, polyamine oxidase 1, and spermidine/spermine N^1-acetyltransferase in RAW 264.7 macrophages. Cells were stimulated with lysate of *H. pylori* at a multiplicity of infection of 100 for 6 h. **(A)** Reverse transcriptase polymerase chain reaction; **(B)** Real-time polymerase chain reaction analysis, using SYBR Green, data are shown on a logarithmic scale. $^{**}p < 0.01$, $^{***}p < 0.001$ vs unstimulated cells. Data shown are from ref. *41*. HPL, lysate of *H. pylori*; ODC, ornithine decarboxylase; PAO1, polyamine oxidase; SSAT, spermidine/spermine N^1-acetyltransferase.

it could induce macrophage apoptosis in vitro when separated from the cells by a filter support *(35)* or when bacterial supernatants or lysed bacteria were used *(41)*. Figure 2 demonstrates that *H. pylori* lysate (HPL) induced increased messenger RNA (mRNA) expression of *ODC*, *SMO(PAO1)*, and *SSAT*, but not *APAO (41)*. ODC activity peaked at 6 h after HPL stimulation and exhibited elevation to 24 h (Fig. 3A). As shown in Fig. 3B, SMO(PAO1) activity was significantly increased at 6 h and continued to increase until its peak at 18 h. In contrast, APAO activity is not induced, and SSAT activity is not detectable until 18 h. The polyamine profile after HPL stimulation (Fig. 3C) indicated that

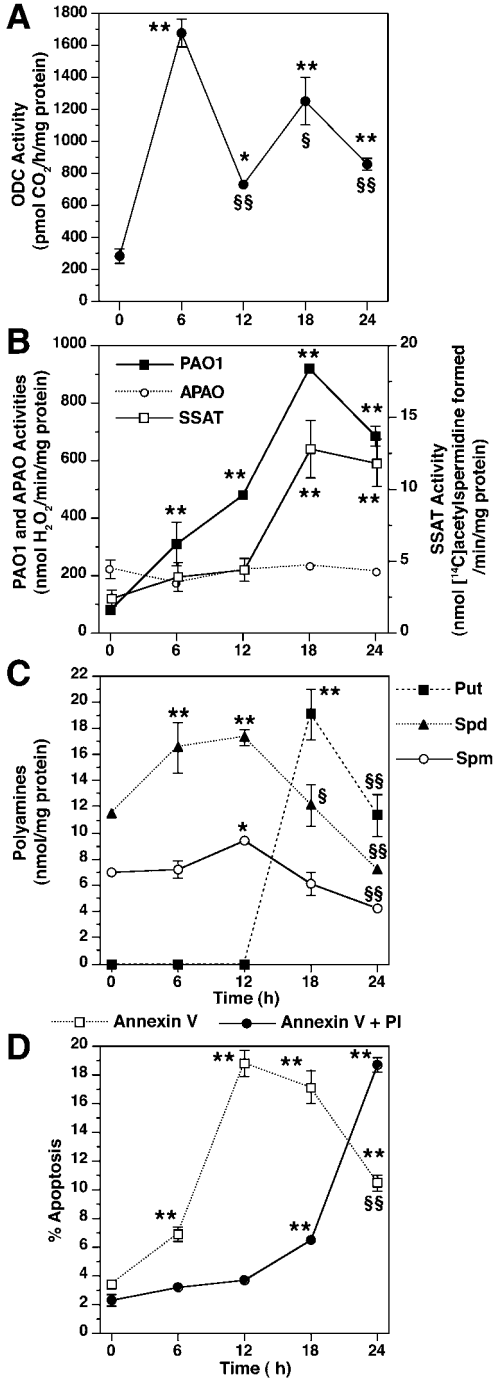
A
ODC Activity
(pmol CO2/h/mg protein)
B
PAO1 and APAO Activities
(nmol H2O2/min/mg protein)
SSAT Activity
(nmol [14C]acetylspermidine formed
/min/mg protein)
PAO1
APAO
SSAT
C
Polyamines
(nmol/mg protein)
Put
Spd
Spm
Time (h)
Annexin V
Annexin V + PI
D
% Apoptosis
Time (h)

Fig. 3

in the first 12 h, all of the putrescine was likely converted to spermidine. The increase in spermidine was significant at 6 and 12 h, and the spermine was increased at 12 h, but less so than spermidine; from 12–24 h, there was a clear decline in spermine and spermidine that was inversely proportional to the increase in putrescine. These data are consistent with the initial induction of SMO(PAO1) followed by the subsequent induction of SSAT activity.

By using annexin V plus PI labeling of live cells and flow cytometry, we measured both early and late apoptosis *(41)*. Figure 3D indicates that early apoptosis, representing annexin V^+/PI^- cells, was significantly increased at 6 and 12 h after HPL stimulation, and as the early apoptosis decreased from 12 to 24 h, there was a concomitant increase in late apoptosis that peaked at 18 h, an indication that early apoptosis represented a progressive apoptotic process. The presence of apoptosis beginning at 6 h was consistent with the initial spike in ODC activity at 6 h and the increase in PAO1 activity at this point, and argues against an important role of the SSAT-APAO pathway in the apoptosis, because SSAT activity did not increase until 18 h after stimulation.

4.4. H. pylori*–Induced Macrophage Apoptosis is Dependent on Spermine Oxidation by SMO(PAO1)*

Because the time course of induction of gene expression and enzyme activity of SMO(PAO1) and polyamine back conversion correlated with apoptosis, we determined the effect of an inhibitor of PAO, MDL 72527. As shown in Fig. 4, the increase in apoptosis and reduction in cell viability with HPL stimulation was inhibited in a concentration-dependent manner by MDL 72527. Similar results were observed in nontransformed mouse peritoneal macrophages *(41)*. Because MDL 72527 inhibits both APAO and SMO(PAO1), we transiently transfected RAW 264.7 cells with a small interfering RNA (siRNA) specific for *SMO(PAO1)*. As shown in Fig. 5, *PAO1* siRNA markedly decreased the HPL-stimulated PAO1 mRNA expression (Fig. 5A) and completely blocked the induction of PAO1 enzyme activity (Fig. 5B). In parallel, the *PAO1* siRNA completely abolished the HPL-induced apoptosis (Fig. 5C), and restored the cell viability (Fig. 5D). Because knockdown of stimulated PAO1 expression completely eliminated HPL-induced macrophage apoptosis, we have provided direct evidence that SMO(PAO1), specifically, is responsible for the apoptosis *(41)*.

Fig. 3. Time course of induction of enzyme activities of ODC, PAO1, SSAT, and associated changes in polyamine levels and apoptosis. RAW 264.7 macrophages were stimulated with HPL at multiplicity of infection of 100. In **(A)**, ODC activity was measured by conversion of [^{14}C]L-ornithine to [^{14}C]O_2. In **(B)**, PAO1 and APAO activities were measured by the luminol method, assessing liberation of H_2O_2 from either spermine in the case of PAO1, or N^1-acetylspermine, for APAO. SSAT activity was determined by incorporation of L-[^{14}C]acetylCoA into spermidine and formation of L-[^{14}C]acetylspermidine. In **(C)**, polyamine levels were determined by HPLC from macrophage lysates. In **(D)**, live cells were stained with annexin V (FITC-labeled) and propidium iodide (PI) and flow cytometry performed. Summary data demonstrating peak early apoptosis (annexin V positive only) at 12 h, and peak late apoptosis (both annexin V and PI positive) at 24 h. *$p < 0.05$, **$p < 0.01$ vs time 0, §$p < 0.05$, §§$p < 0.01$ vs peak level. (Data shown are from ref. *41*.)

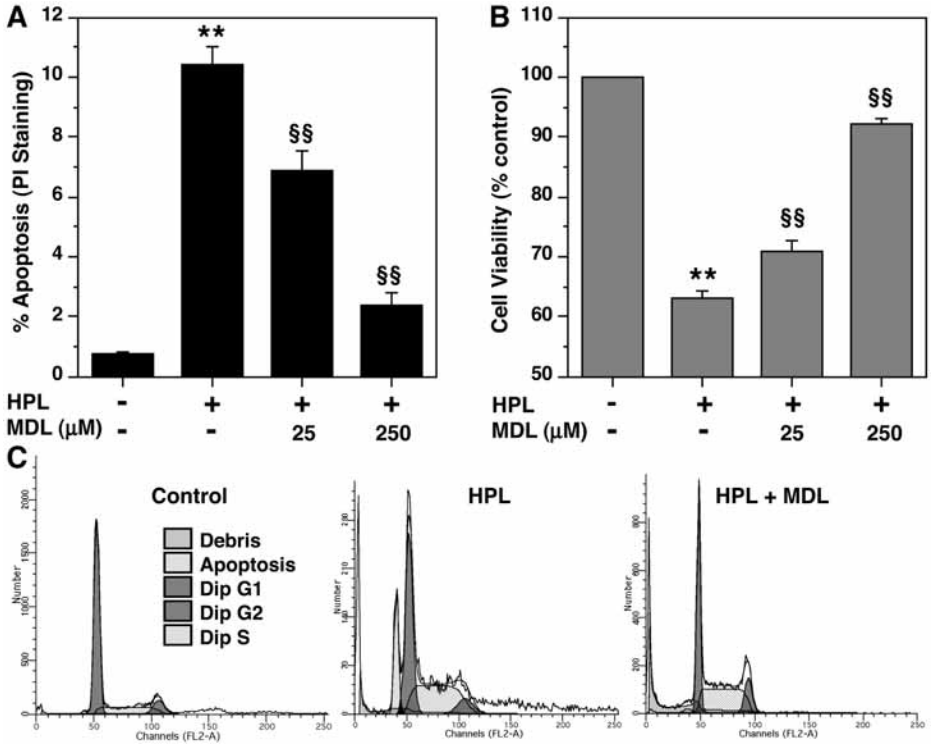

Fig. 4. Inhibition of *H. pylori*-induced apoptosis in macrophages by the PAO inhibitor MDL 72527. **(A)** Summary data of apoptosis in RAW 264.7 cells stimulated with *H. pylori* lystate (HPL), determined by propidium iodide (PI) staining of fixed cells followed by flow cytometry and quantification of apoptosis by ModFit-LT software. **(B)** Demonstration that HPL reduces cell viability, determined by XTT assay, with HPL, and inhibition of this effect with MDL 72527 in concordance with data in **(A)**. **(C)** Representative histogram plots of PI stained cells (data summarized in **[A]**) showing the increase in cells in the sub-G_0/G_1 fraction, indicative of apoptosis, with HPL stimulation, and inhibition with MDL 72527. $^{**}p < 0.01$ vs unstimulated cells, §§$p < 0.01$ vs HPL only. (Data shown are from ref. *41*.)

4.5. Overexpression of SMO(PAO1) Causes Macrophage Apoptosis

When RAW 264.7 cells were transfected with a full-length complimentary DNA for *SMO(PAO1)*, there was a 7.3 ± 0.9-fold increase in SMO(PAO1) activity that was paralleled by a 7.2 ± 0.5-fold increase in apoptosis; the level of enzyme activity and apoptosis so generated was essentially identical to that induced by HPL in the neo-transfected cells *(41)*.

4.6. H. pylori*–Induced SMO(PAO1) Results in H_2O_2 Generation That Causes Apoptosis in Macrophages*

To determine if H_2O_2 released by SMO(PAO1) activation was responsible for the apoptosis, we measured intracellular H_2O_2 levels in response to HPL by flow cytometry.

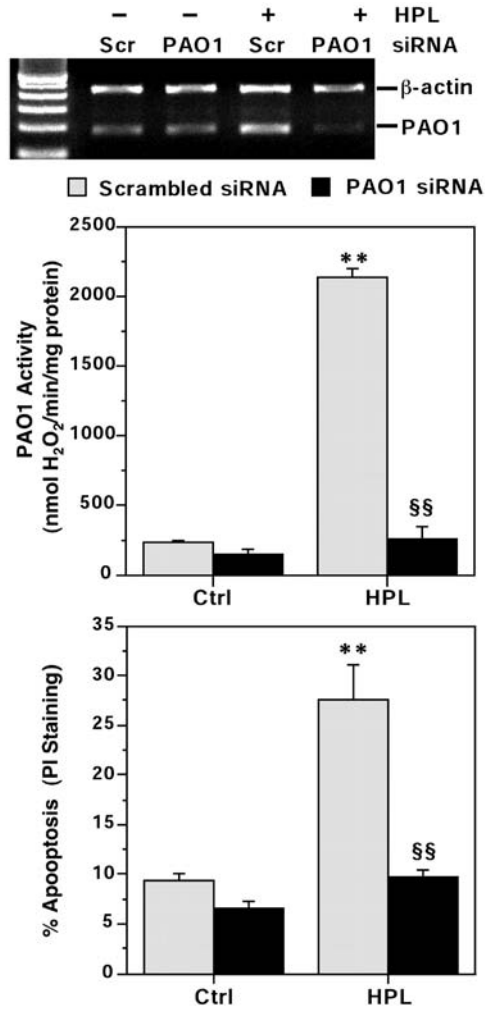

Fig. 5. Transfection with *PAO1* siRNA inhibits *H. pylori*-stimulated mRNA expression, enzyme activity, and apoptosis in macrophages. RAW 264.7 cells were transiently transfected with duplex small interfering RNA (siRNA) targeted against nt 467–487 in the coding sequence for murine *PAO1* or with scrambled siRNA control. **(A)** Reverse transcriptase polymerase chain reaction analysis of cells transfected as indicated, in the absence and presence of HPL for 6 h. **(B)** PAO1 enzyme activity measured as in Fig. 2B, after 24 h. **(C)** apoptosis, measured by PI staining and flow cytometry of fixed cells. **(D)** Cell viability, measured by XTT assay. $^{**}p < 0.01$ vs scrambled siRNA alone without HPL; §§$p < 0.01$ for PAO1 siRNA vs scrambled siRNA. (Data shown are from ref. *41*.)

As shown in Fig. 6A, HPL induced a significant increase that was attenuated by MDL 72527 or the H_2O_2-detoxifying enzyme, catalase. We also used the Amplex Red® assay that specifically measures H_2O_2 in solution to demonstrate that supernatant levels of

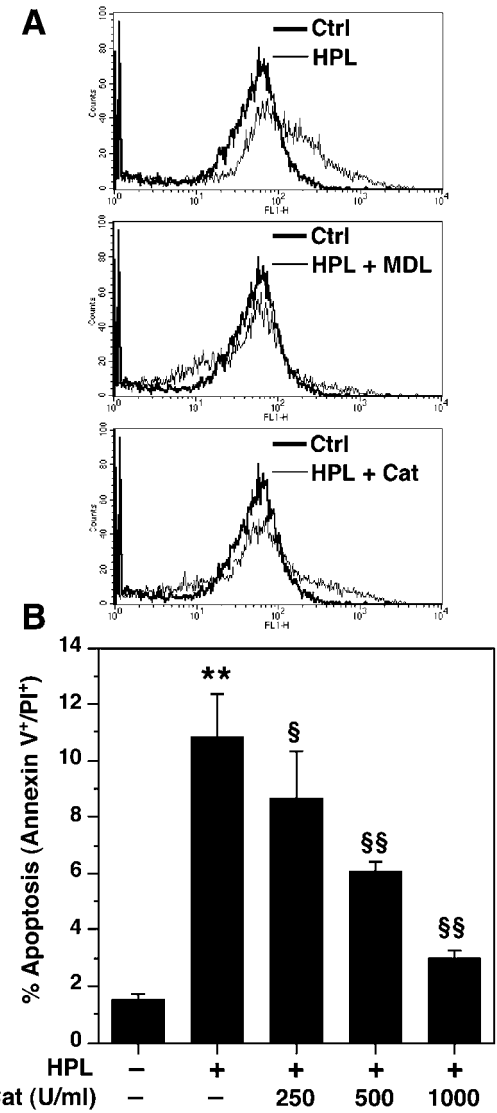

Fig. 6. *H. pylori* induces H_2O_2 production in RAW 264.7 macrophages, that is inhibited by MDL 72527, and catalase, and inhibition of apoptosis with catalase. Cells were stimulated with HPL and live macrophages were treated with CM-H_2DCFDA; representative fluorescence data are shown. The thick line represents the fluorescence tracing from unstimulated cells. The thin line represents the fluorescence tracing from the cells stimulated with HPL alone or with HPL plus MDL 72527 or catalase. Note the shift of the curve to the right with HPL, indicating more fluorescence intensity, which is prevented by MDL 72527 or catalase. **(B)** Catalase inhibits apoptosis in RAW 264.7 macrophages, as assessed by staining with PI and annexin V. $^{**}p < 0.01$ vs control, §$p < 0.05$, §§$p < 0.01$ vs HPL alone. (Data shown are from ref. *41*.)

H_2O_2 induced by HPL were markedly inhibited by MDL 72527 or catalase. Consistent with these findings, catalase inhibited apoptosis in a concentration-dependent manner (Fig. 6B). Cell-permeable polyethylene glycolated-catalase was able to inhibit the apoptosis by 100% *(41)*. These studies indicate that H_2O_2 generation is a major cause of HPL-induced apoptosis.

4.7. SMO(PAO1) Induction Results in Mitochondrial Membrane Depolarization, Cytochrome c Release, and Caspase-3 Activation

Because depolarization of mitochondrial membrane potential ($\Delta\psi_m$) has been implicated in apoptosis, we determined the ability of *H. pylori* to cause this event *(41)*. As shown in Fig. 7A, there was a significant decrease in $\Delta\psi_m$ that was significantly inhibited by MDL 72527. To confirm activation of the mitochondrial apoptosis pathway, release of cytochrome c from mitochondria to cytosol was assessed by immunoblotting. As shown in Fig. 7B, with HPL stimulation there was a significant decrease in mitochondrial cytochrome c, and a concomitant increase in cytoplasmic cytochrome c, indicating translocation from mitochondria to cytosol. In cells treated with MDL 72527, there was inhibition of the decrease in mitochondrial levels and the increase in cytosolic levels of cytochrome c, indicating the prevention of the translocation. These findings were confirmed by immunohistochemistry *(41)*. Cytochrome c released from mitochondria activates caspase-9, which ultimately activates caspase-3, a final step in activation of exonucleases and apoptosis *(42)*. After activation with HPL, macrophage caspase-3 activity increased significantly in a time-dependent manner (Fig. 7C) that paralleled that of the late apoptosis (Fig. 3D). As shown in Fig. 7D, MDL 72527 significantly reduced caspase-3 activity.

4.8. Proposed Mechanism of H. pylori*–Induced Apoptosis by Polyamines*

Figure 8 provides a schematic of interpretation of our findings that *H. pylori* itself or soluble products derived from the bacterium induce apoptosis caused by expression of ODC and SMO(PAO1). ODC generates putrescine that is converted into spermidine and spermine. The latter is back converted by the increased levels of SMO(PAO1) to spermidine, releasing H_2O_2 that causes depolarization of the mitochondrial membrane, release of cytochrome c to the cytosol, and activation of caspase-3, leading to apoptosis.

5. Additional Aspects of the Induction of Macrophage Apoptosis by Polyamines

5.1. Specificity of Response

Because macrophages exposed to stress, such as with *H. pylori* infection, undergo a vast array of innate responses, it is possible that the induction of apoptosis itself could be activating polyamine synthesis or oxidative catabolism. However, we have determined that when apoptosis is inhibited by catalase or by cyclosporine A (which blocks mitochondrial membrane depolarization), the induction of ODC or SMO(PAO1) by *H. pylori* is not prevented, and when apoptosis is induced by another bacterial stimulus, *Citrobacter rodentium* (which causes colitis in mice) or a chemical stimulus, staurosporine, ODC, and PAO1 are not induced (Y. Cheng, R. Chaturvedi, and K. T. Wilson, unpublished data). These data indicate that the response to *H. pylori* may be specific,

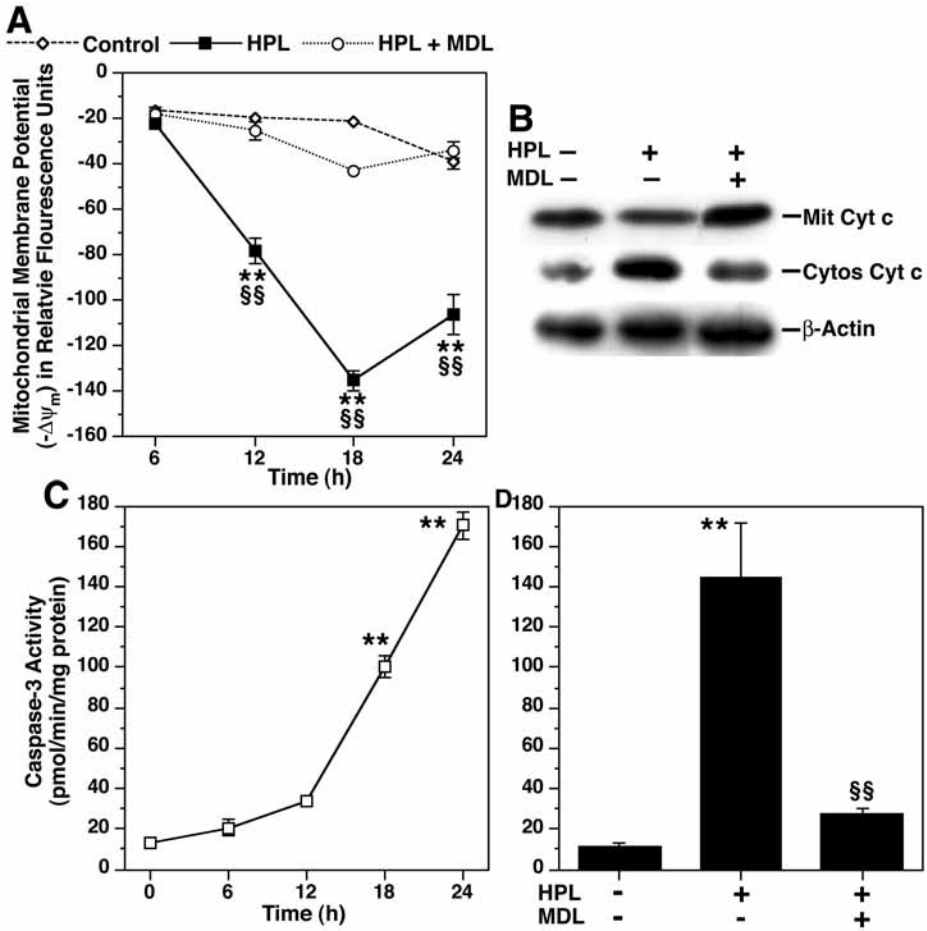

Fig. 7. *H. pylori* induces mitochondrial membrane depolarization, translocation of cytochrome c from mitochondria to cytosol, and activation of caspase-3 that is inhibited by MDL 72527. RAW 264.7 cells were treated with HPL in the absence and presence of MDL 72527 (250 μ*M*). **(A)** Mitochondrial membrane potential was measured by flow cytometry using MitoCapture dye at the times indicated. Summary data of mean relative fluorescence, expressed as negative values to indicate depolarization is shown. $^{**}p < 0.01$ vs control, §§$p < 0.01$ vs HPL + MDL 72527. **(B)** Western blot analysis for cytochrome c (15 kDA protein). Upper panel: mitochondrial fraction; middle panel: cytosolic fraction. Equal amounts of protein were loaded per lane, and equal loading was verified by staining of membranes with Ponceau S and immunoblotting for β-actin (lower panel). **(C)** Cells were treated with HPL and caspase-3 activity measured by colorimetric assay at the times indicated. **(D)** In cells assessed at 24 h after stimulation, MDL 72527 (250 μ*M*) blocked the caspase-3 activation. For **(C)** and **(D)**, $^{**}p < 0.01$ vs control, §§$p < 0.01$ vs HPL. (Data shown are from ref. *41*.)

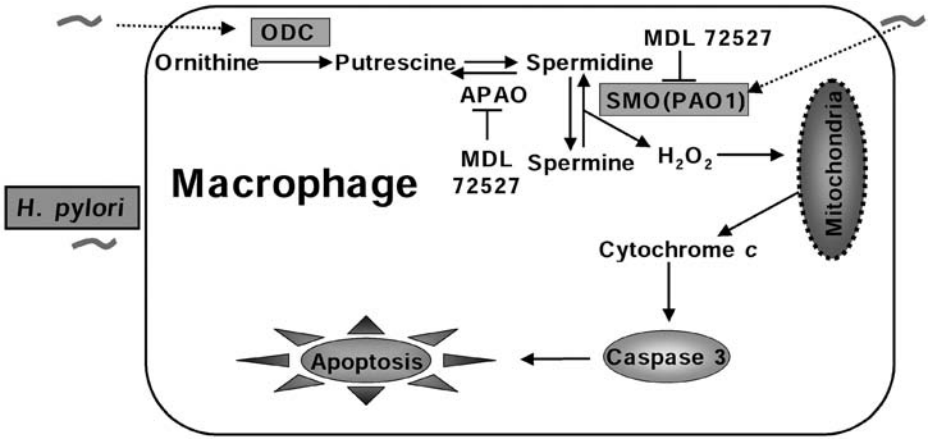

Fig. 8. Proposed mechanism of *H. pylori*-induced macrophage apoptosis in macrophages by polyamines. *H. pylori* stimulates expression of ODC, generating polyamines, and the increased spermine is acted upon by SMO(PAO1) that is also upregulated by *H. pylori*. The H_2O_2 so generated causes mitochondrial membrane depolarization, release of cytochrome c from mitochondria to cytosol, and activation of caspase-3, leading to apoptosis.

and additional studies are in progress in our laboratory to determine the response to other pathogenic stimuli.

5.2. Further Insights Into the Induction of Macrophage Apoptosis by Polyamines

Previous work from our laboratory has implicated specific *H. pylori*-derived factors in the activation of macrophages, including urease *(3)*. This enzyme serves a biochemically essential function for the bacterium of converting urea to ammonia and carbon dioxide, creating an alkaline microenvironment required for the organism to survive in the acid milieu of the stomach. However, urease is also an immunologically active protein, and we have found that urease appears to mediate the induction of ODC in macrophages because isogenic mutant strains of *H. pylori* lacking urease exhibited significant attenuation of induction of ODC expression and activity, and recombinant urease stimulated ODC activation *(43)*.

ODC expression has been shown to be enhanced by binding of the transcription factor c-Myc to the ODC promoter *(6)*. We have substantial evidence that *H. pylori* and its urease induces ODC by a c-Myc-dependent process, because *H. pylori* induces c-Myc expression and inhibition of c-Myc binding prevents activation of the ODC promoter, ODC mRNA expression, ODC activity, and apoptosis *(43)*. Additionally, we have recently found that the activation of c-Myc and induction of ODC in response to *H. pylori* is dependent on phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 (M. Asim, R. Chaturvedi, and K.T. Wilson, manuscript in preparation).

6. Involvement of SMO(PAO1) in the Pathogenic Aspects of Other Model Systems

We have conducted additional studies that indicate that SMO(PAO1) activity is important in other disease states. Specifically, we have recently reported that as in macrophages, SMO(PAO1) expression is upregulated in gastric epithelial cells exposed to *H. pylori* and that inhibition with MDL72527 or *SMO(PAO1)* siRNA significantly attenuated the induction of apoptosis *(44)*. Importantly, we also demonstrated that DNA damage induced by *H. pylori* in these cells was mediated by H_2O_2 derived from induction of SMO(PAO1), which may have direct relevance to *H. pylori*-associated gastric cancer *(44)*. Additionally, we reported that polyamines are beneficial in mouse colitis *(45)*, and we now have evidence that SMO(PAO1) correlates with the severity of disease in several colitis models, suggesting that oxidative stress derived from spermine oxidation may play an important role either from apoptosis or other effects that we are now investigating.

7. The Balance of Spermine and its Oxidation Products, Determined by ODC and SMO(PAO1) is Important in the Regulation of Innate Immune Response

We have previously reported that in a coculture model in which *H. pylori* is separated from macrophages by a filter support the bacteria are killed by a NO-dependent mechanism *(46)*. However, in the stomach, the bacteria persists for the life of the host, despite the expression of iNOS in the gastric mucosa *(39)*. Because spermine has been shown to downregulate cytokine production by lipopolysaccharide-stimulated macrophages *(47)*, we have assessed the effect of spermine on iNOS *(48)*. Spermine inhibited NO production from *H. pylori*-stimulated macrophages in a concentration-dependent manner. Intriguingly, there was no effect on the induction of iNOS mRNA, but rather there was potent inhibition of iNOS protein translation. Consistent with this, knockdown of *ODC* by siRNA increased iNOS protein expression and NO production in response to *H. pylori*. This enhanced killing of the bacterium, whereas spermine inhibited killing *(48)*. Recently, we have found that knockdown of *SMO(PAO1)* in *H. pylori*-stimulated macrophages prevents depletion of spermine, and thus inhibits iNOS protein levels, NO production, and bacterial killing (R. Chaturvedi and K.T. Wilson, manuscript in preparation).

8. Summary

Evidence has been presented in this review indicating that polyamines can have either a pro- or antiapoptotic effect in immune cells. This most likely depends greatly on the inducing stimuli, the cell type, and other factors in the intracellular environment. We have presented our work in the *H. pylori* model that has identified a pathway of macrophage apoptosis in which the activation of arginase, ODC, and SMO(PAO1) is required, with the latter resulting in generation of H_2O_2 that activates the mitochondrial pathway of apoptosis. We suggest that the balance of spermine and its oxidation is a critical aspect of innate immune response to pathogens, in that spermine accumulation inhibits responses such as iNOS-derived NO and cytokine production, whereas spermine catabolism leads to oxidative stress, apoptosis, and DNA damage.

9. Acknowledgments

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References

1. Akira, S., Takeda, K., and Kaisho, T. (2001) Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat. Immunol.* **2,** 675–680.
2. Panchal, P. C., Forman, J. S., Blumberg, D. R., and Wilson, K. T. (2003) *Helicobacter pylori* infection: pathogenesis. *Curr. Opin. Gatroenterol.* **19,** 4–10.
3. Gobert, A. P., Mersey, B. D., Cheng, Y., Blumberg, D. R., Newton, J. C., and Wilson, K. T. (2002) Cutting edge: Urease release by *Helicobacter pylori* stimulates macrophage inducible nitric oxide synthase. *J. Immunol.* **168,** 6002–6006.
4. Ina, K., Itoh, J., Fukushima, K., et al. (1999) Resistance of Crohn's disease T cells to multiple apoptotic signals is associated with a Bcl-2/Bax mucosal imbalance. *J. Immunol.* **163,** 1081–1090.
5. Zychlinsky, A., Prevost, M. C., and Sansonetti, P. J. (1992) Shigella flexneri induces apoptosis in infected macrophages. *Nature* **358,** 167–169.
6. Packham, G. and Cleveland, J. L. (1994) Ornithine decarboxylase is a mediator of c-Myc-induced apoptosis. *Mol. Cell Biol.* **14,** 5741–5747.
7. Li, L., Rao, J. N., Bass, B. L., and Wang, J. Y. (2001) NF-kappaB activation and susceptibility to apoptosis after polyamine depletion in intestinal epithelial cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* **280,** G992–G1004.
8. Maccarrone, M., Bari, M., Battista, N., Di Rienzo, M., Falciglia, K., and Finazzi Agro, A. (2001) Oxidation products of polyamines induce mitochondrial uncoupling and cytochrome c release. *FEBS Lett.* **507,** 30–34.
9. Ghafourifar, P., Klein, S. D., Schucht, O., et al. (1999) Ceramide induces cytochrome c release from isolated mitochondria. Importance of mitochondrial redox state. *J. Biol. Chem.* **274,** 6080–6084.
10. Green, D. R. and Reed, J. C. (1998) Mitochondria and apoptosis. *Science* **281,** 1309–1312.
11. Yuan, Q., Ray, R. M., and Johnson, L. R. (2002) Polyamine depletion prevents camptothecin-induced apoptosis by inhibiting the release of cytochrome c. *Am. J. Physiol. Cell Physiol.* **282,** C1290–1297.
12. Babbar, N., Ignatenko, N. A., Casero, R. A., Jr., and Gerner, E. W. (2003) Cyclooxygenase-independent induction of apoptosis by sulindac sulfone is mediated by polyamines in colon cancer. *J. Biol. Chem.* **278,** 47,762–47,775.
13. Bock, J. M., Pickart, M. A., Pink, J. J., and Harari, P. M. (1999) Modulation of tumor cell proliferation and apoptosis by polyamine depletion in cells of head and neck squamous cell carcinomas. *Radiat. Res.* **152,** 604–610.
14. Choi, S. H., Kim, S. W., Choi, D. H., Min, B. H., and Chun, B. G. (2000) Polyamine-depletion induces p27Kip1 and enhances dexamethasone-induced G1 arrest and apoptosis in human T lymphoblastic leukemia cells. *Leuk. Res.* **24,** 119–127.
15. Takahashi, Y., Mai, M., and Nishioka, K. (2000) alpha-Difluoromethylornithine induces apoptosis as well as anti-angiogenesis in the inhibition of tumor growth and metastasis in a human gastric cancer model. *Int. J. Cancer* **85,** 243–247.
16. Penning, L. C., Schipper, R. G., Vercammen, D., et al. (1998) Sensitization of tnf-induced apoptosis with polyamine synthesis inhibitors in different human and murine tumour cell lines. *Cytokine* **10,** 423–431.

17. Choi, W., Gerner, E. W., Ramdas, L., et al. (2005) Combination of 5-fluorouracil and N1, N11-diethylnorspermine markedly activates spermidine/spermine N1-acetyltransferase expression, depletes polyamines, and synergistically induces apoptosis in colon carcinoma cells. *J. Biol. Chem.* **280,** 3295–3304.
18. Shirahata, A. and Pegg, A. E. (1985) Regulation of S-adenosylmethionine decarboxylase activity in rat liver and prostate. *J. Biol. Chem.* **260,** 9583–9588.
19. Hegardt, C., Andersson, G., and Oredsson, S. M. (2000) Changes in polyamine metabolism during glucocorticoid-induced programmed cell death in mouse thymus. *Cell Biol. Int.* **24,** 871–880.
20. Mather, M. and Rottenberg, H. (2001) Polycations induce the release of soluble intermembrane mitochondrial proteins. *Biochim. Biophys. Acta* **1503,** 357–368.
21. Parchment, R. E. (1991) Programmed cell death (apoptosis) in murine blastocysts: extracellular free-radicals, polyamines, and other cytotoxic agents. *In Vivo* **5,** 493–500.
22. Ha, H. C., Woster, P. M., Yager, J. D., and Casero, R. A., Jr. (1997) The role of polyamine catabolism in polyamine analogue-induced programmed cell death. *Proc. Natl. Acad. Sci. USA* **94,** 11,557–11,562.
23. Casero, R. A., Jr., Celano, P., Ervin, S. J., Applegren, N. B., Wiest, L., and Pegg, A. E. (1991) Isolation and characterization of a cDNA clone that codes for human spermidine/spermine N1-acetyltransferase. *J. Biol. Chem.* **266,** 810–814.
24. Casero, R. A., Jr. and Pegg, A. E. (1993) Spermidine/spermine N1-acetyltransferase—the turning point in polyamine metabolism. *FASEB J.* **7,** 653–661.
25. Vujcic, S., Liang, P., Diegelman, P., Kramer, D. L., and Porter, C. W. (2003) Genomic identification and biochemical characterization of the mammalian polyamine oxidase involved in polyamine back-conversion. *Biochem. J.* **370,** 19–28.
26. Niiranen, K., Pietila, M., Pirttila, T. J., et al. (2002) Targeted disruption of spermidine/spermine N1-acetyltransferase gene in mouse embryonic stem cells. Effects on polyamine homeostasis and sensitivity to polyamine analogues. *J. Biol. Chem.* **277,** 25,323–25,328.
27. Wang, Y., Devereux, W., Woster, P. M., Stewart, T. M., Hacker, A., and Casero, R. A., Jr. (2001) Cloning and characterization of a human polyamine oxidase that is inducible by polyamine analogue exposure. *Cancer Res.* **61,** 5370–5373.
28. Vujcic, S., Diegelman, P., Bacchi, C. J., Kramer, D. L., and Porter, C. W. (2002) Identification and characterization of a novel flavin-containing spermine oxidase of mammalian cell origin. *Biochem. J.* **367,** 665–675.
29. Devereux, W., Wang, Y., Stewart, T. M., et al. (2003) Induction of the PAOh1/SMO polyamine oxidase by polyamine analogues in human lung carcinoma cells. *Cancer Chemother. Pharmacol.* **52,** 383–390.
30. Ferioli, M. E., Pinotti, O., and Pirona, L. (1999) Polyamine oxidase activity in lymphoid tissues of glucocorticoid-treated rats. *Biochem. Pharmacol.* **58,** 1907–1914.
31. Ferioli, M. E., Bottone, M. G., Soldani, C., and Pellicciari, C. (2004) Administration of the antitumor drug mitoguazone protects normal thymocytes against spontaneous and etoposide-induced apoptosis. *Cell Mol. Life Sci.* **61,** 2767–2773.
32. Jan, M. S., Wing, L. Y., Lin, M. T., and Lin, Y. S. (1999) alpha-Difluoromethylornithine blocks thymocyte apoptosis via a reduction in tyrosine phosphorylation. *Scand. J. Immunol.* **50,** 605–611.
33. Lindsay, G. S. and Wallace, H. M. (1999) Changes in polyamine catabolism in HL-60 human promyelogenous leukaemic cells in response to etoposide-induced apoptosis. *Biochem. J.* **337,** 83–87.

34. Monti, M. G., Ghiaroni, S., Pernecco, L., Barbieri, D., Marverti, G., and Franceschi, C. (1998) Polyamine depletion protects HL-60 cells from 2-deoxy-D-ribose-induced apoptosis. *Life Sci.* **62,** 799–806.
35. Gobert, A. P., Cheng, Y., Wang, J. Y., et al. (2002) *Helicobacter pylori* induces macrophage apoptosis by activation of arginase II. *J. Immunol.* **168,** 4692–4700.
36. Stefanelli, C., Bonavita, F., Stanic, I., et al. (1998) Spermine causes caspase activation in leukaemia cells. *FEBS Lett.* **437,** 233–236.
37. Nitta, T., Igarashi, K., Yamashita, A., Yamamoto, M., and Yamamoto, N. (2001) Involvement of polyamines in B cell receptor-mediated apoptosis: Spermine functions as a negative modulator. *Exp. Cell Res.* **265,** 174–183.
38. Nitta, T., Igarashi, K., and Yamamoto, N. (2002) Polyamine depletion induces apoptosis through mitochondria-mediated pathway. *Exp. Cell Res.* **276,** 120–128.
39. Fu, S., Ramanujam, K. S., Wong, A., et al. (1999) Increased expression and cellular localization of inducible nitric oxide synthase and cyclooxygenase 2 in *Helicobacter pylori* gastritis. *Gastroenterol.* **116,** 1319–1329.
40. Albina, J. E., Cui, S., Mateo, R. B., and Reichner, J. S. (1993) Nitric oxide-mediated apoptosis in murine peritoneal macrophages. *J. Immunol.* **150,** 5080–5085.
41. Chaturvedi, R., Cheng, Y., Asim, M., et al. (2004) Induction of polyamine oxidase 1 by *Helicobacter pylori* causes macrophage apoptosis by hydrogen peroxide release and mitochondrial membrane depolarization. *J. Biol. Chem.* **279,** 40,161–40,173.
42. Nicholson, D. W., Ali, A., Thornberry, N. A., et al. (1995) Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* **376,** 37–43.
43. Cheng, Y., Chaturvedi, R., Yao, M., et al. (2004) Urease induces macrophage apoptosis by c-Myc-mediated activation of ornithine decarboxylase (ODC). *Gastroenterol.* **126,** A108.
44. Xu, H., Chaturvedi, R., Cheng, Y., et al. (2004) Spermine oxidation induced by *Helicobacter pylori* results in apoptosis and DNA damage: Implications for gastric carcinogenesis. *Cancer Res.* **64,** 8521–8525.
45. Gobert, A. P., Cheng, Y., Akhtar, M., et al. (2004) Protective role of arginase in a mouse model of colitis. *J. Immunol.* **173,** 2109–2117.
46. Gobert, A. P., McGee, D. J., Akhtar, M., et al. (2001) *Helicobacter pylori* arginase inhibits nitric oxide production by eukaryotic cells: A strategy for bacterial survival. *Proc. Natl. Acad. Sci. USA* **98,** 13,844–13,849.
47. Zhang, M., Caragine, T., Wang, H., et al. (1997) Spermine inhibits proinflammatory cytokine synthesis in human mononuclear cells: a counterregulatory mechanism that restrains the immune response. *J. Exp. Med.* **185,** 1759–1768.
48. Bussiere, F. I., Chaturvedi, R., Cheng, Y., et al. (2005) Spermine causes loss of innate immune response to *Helicobacter pylori* by inhibition of inducible nitric-oxide synthase translation. *J. Biol. Chem.* **280,** 2409–2412.

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Polyamines and Cancer

Ajit K. Verma

1. Introduction

The polyamines putrescine, spermidine, and spermine are aliphatic cations that play important roles in normal cell proliferation and differentiation and have been shown to be constitutively increased during malignant transformation *(1,2)*. Polyamines are ubiquitous in nature and their cellular levels are intricately regulated by both biosynthetic (ornithine decarboxylase (ODC), *S*-adenosylmethionine decarboxylase, spermidine synthase, and spermine synthase) and catabolic enzymes (spermidine/spermine acetyl transferase, flavin containing polyamine oxidase, copper containing diamine oxidase, and other amine oxidases) (Fig. 1). Increased levels of polyamines, as observed in human cancers, are the result of deregulation of polyamine metabolism and transport *(3,4)*. Consequently, the polyamine biosynthetic, degradative, and transport pathways have been explored as potential targets for both cancer prevention and therapeutic interventions *(5–8)*. Evidence is presented in this chapter indicating that signaling networks that regulate polyamine levels are possible targets for the design of drugs for human cancer prevention and treatment.

2. ODC, the Key Enzyme of Mammalian Polyamine Biosynthesis, Linked to Cancer Induction and Progression

2.1. ODC Induction is an Essential Component of Tumor Promotion

As evidenced in both humans and experimental animals, the process of cancer formation involves multiple steps: initiation, promotion, and progression (Fig. 2) *(9,10)*.

Although development of cancer can be prevented by intervention at any one of the steps involved in the induction of cancer, interference with the promotion stage of carcinogenesis appears to be most appropriate and practical. The rationale behind this comes from the facts that promotion of tumor formation requires a repeated and prolonged exposure to a promoter, and that tumor promotion is reversible, at least in the early stages. In contrast, initiation can be accomplished by a single exposure to a sufficiently small dose of a carcinogen, and this step is rapid and irreversible. Because

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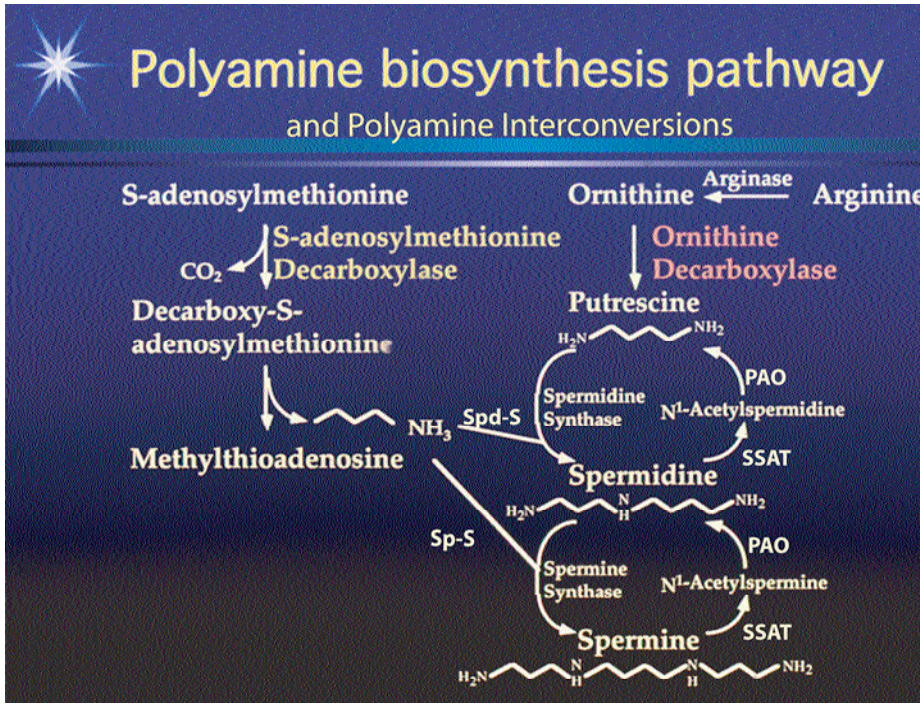


Fig. 1. Polyamine biosynthesis and catabolism. Spd-S, spermidine synthase, Sp-S, spermine synthase, PAO, polyamine oxidase, SSAT, spermidine/spermine N^1-acetyltransferase.

humans are constantly exposed to environmental carcinogens, and that the human diet contains nitrites and nitrates, which are converted in the gut to potent carcinogen nitrosamines, one may believe that the initiation step of carcinogenesis is inevitable. Furthermore, progression, a critical and late step of carcinogenesis, which involves conversion of a preneoplastic cell to a cancer cell within a benign tumor, involves additional genetic events and is not clearly defined. Thus cancer prevention strategies should be based on knowledge of the mechanism of the promotion step of oncogenesis. The multistep model of mouse skin carcinogenesis has been on the forefront of the identification of irreversible genetic events of initiation and progression, and epigenetic events of tumor promotion *(9,10)*. The type of preneoplastic lesions that develop first in mouse skin with initiation with 7,12-dimethylbenz[a]anthracene (DMBA) and promotion with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) are skin papillomas. The papillomas are benign lesions, some of them regress and others persist and a few develop into invasive squamous cell carcinomas (SCC).

Chronic exposure to ultraviolet radiation (UVR) is the most common etiological factor linked to the development of SCC and basal cell carcinomas (BCC), the most common nonmelanoma forms of human skin cancer. UVA (315–400 nm), UVB (280–315 nm), and UVC (190–280 nm) are the three components of the UV spectrum

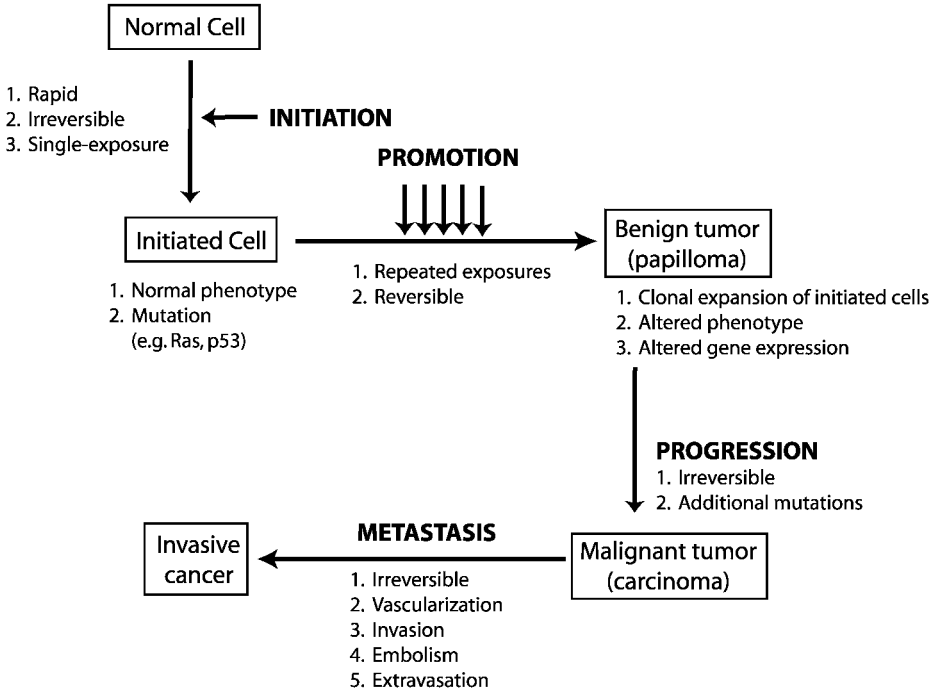


Fig. 2. Multistep carcinogenesis.

(11). Since stratospheric ozone absorbs most of the radiation below 310 nm (UVC), the UVR that reaches us on earth comprises mostly UVA (90–99%) and UVB (1–10%). UVA and UVB are the most prominent and ubiquitous carcinogenic wavelengths in our natural environment *(11,12)*. SCC, unlike BCC, invades the nearby tissues. The first site of metastasis usually is a regional lymph node before metastatic growth in distant sites, such as the lung and brain.

Both TPA and the tumor promotion component of UVR carcinogenesis involve clonal expansion of the initiated cells and result in aberrant expression of genes altered during tumor initiation. TPA and UVR have been reported to alter the expression of genes regulating inflammation, cell growth, and differentiation. Specific examples include upregulation of the expression of p21 (WAF1/C1P1), p53, AP-1 activation, ODC, cyclo-oxygenase-2, tumor necrosis factor (TNF)-a, and a wide variety of cytokines and growth factors *(13)*. Available data indicate that the induction of ODC activity, and the resultant accumulation of putrescine, are essential components of the mechanism of tumor promotion *(7)*.

ODC, which decarboxylates ornithine to putrescine, is characterized by its inducibility and rapid turnover rate (half-life approx 17 min). ODC is the key enzyme in mammalian polyamine biosynthesis. Tumor promoter-induced ODC activity always accompanied increase accumulation of putrescine and spermidine, but spermine levels

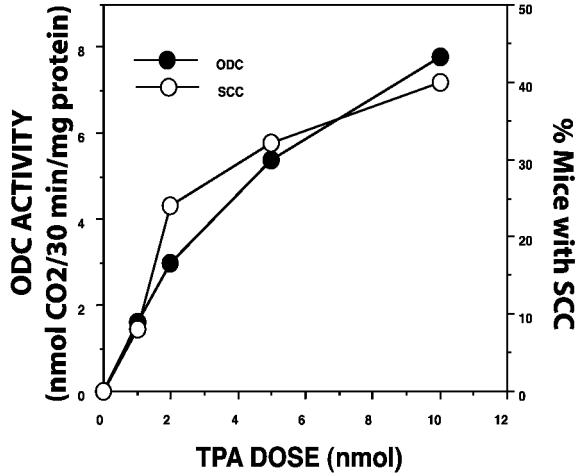


Fig. 3. A correlation between induction of ornithine decarboxylase and squamous cell carcinoma by different tumor promoter TPA doses. Female CD1 mice were initiated with 200 nmol DMBA. Two wk after initiation, all mice were promoted twice weekly with different doses of TPA. Carcinoma incidence was determined at 50 wk of TPA promotion. A separate group of mice were treated with the same doses of TPA, soluble epidermal ODC activity was determined 4.5 h after treatment.

remained unaltered. The role of ODC has been extensively analyzed in mouse skin tumor promotion by TPA *(5–7)*. Topical application of TPA to the shaved back of a mouse leads to approximately a 200-fold increase in epidermal ODC activity at 4.5 h after treatment. The magnitude of ODC induction is dose dependent and correlates with the ability of the dose to promote skin tumor formation. The degree of induction of ODC activity correlates well with the tumor-promoting ability of a number of structurally unrelated tumor promoters. Furthermore, the ability of a series of phorbol esters to induce ODC activity correlates with their ability to promote skin tumor formation. Epidermal ODC activity is induced only after treatment of mouse skin with tumor promoters and not after treatment with nonpromoting hyperplastic agents. In contrast, both tumor promoters and hyperplastic agents induce *S*-adenosyl-L-methionine decarboxylase activity, an enzyme involved in the biosynthesis of spermidine and spermine. The latter induction may be related to the hyperplasia commonly observed after their applications to mouse skin. An example indicating an association between ODC induction and skin tumor promotion by TPA is illustrated in Fig. 3.

Proportionality between the maximum degree of induction of ODC activity and the number of carcinoma incidence caused by increasing doses of TPA was observed. These findings have led to a conclusion that the induction of mouse epidermal ODC activity may be a rapid screen for new tumor promoters. In fact, Fujiki et al. *(14)* employed induction of mouse epidermal ODC as one of the rapid tests for the

identification of new classes of naturally occurring tumor promoters, such as okadaic acid. A tight link between increased ODC activity and cancer induction prompted the synthesis of an inhibitor of ODC, a-difluoromethylornithine (DFMO).

2.2. DFMO, a Suicide Inhibitor of ODC

DFMO (Fig. 4), a specific irreversible inhibitor of ODC, precludes accumulation of putrescine and spermidine. DFMO is enzymatically decarboxylated and generates an intermediate carbanionic species that, with the loss of fluorine, alkylates a nucleophilic residue at or near the active site, thereby covalently binding the inhibitor to the enzyme.

DFMO is a water-soluble compound and can be administered intraperitoneally or in drinking water. DFMO given in drinking water inhibits the induction of cancers of skin, breast, colon, intestines, and bladder in experimental animals *(15,16)*. The plasma half-life of DFMO in rat, rabbit, dog, monkey, and healthy man has been quantified, and it varies with species from 83 to 353 min. Treatment of animals with high doses of DFMO for a long period results in several side effects, such as suppression of early embryogenesis and arrest of embryonic development, enhanced ovulation in rats, weight loss, diarrhea, thrombocytopenia, and impairment of the development of the brain in preweanling rats. However, such toxic side effects of DFMO were not observed in mice kept for 32 wk on 0.25% DFMO in the drinking water. Toxicity associated with high doses of DFMO in humans is thrombocytopenia and reversible ototoxicity *(17,18)*. DFMO in combination with cyclo-oxygenase-2 inhibitor was a potent therapeutic agent for cancer *(19)*. Aerosol administration of DFMO was effective in the prevention of cancer of the upper respiratory tract of the Syrian golden hamster *(20)*. Also, topical DFMO reduced the number of human actinic keratoses *(21)*.

2.3. To Define a Link Between Polyamines and Cancer Using Transgenic Mouse Models

Several transgenic mouse models overexpressing ODC or polyamine catabolizing enzymes have been generated to link ODC activity and polyamine level to the induction of skin cancer. Halmekyto et al. were first to generate transgenic mice overexpressing the human *ODC* gene under its own promoter *(22)*. In this ODC-overexpressing transgene, mouse model ODC was overexpressed in all the tissues of the transgenic mice. These ODC-overexpressing transgenic mice elicited enhanced susceptibility to the induction of skin papillomas by the DMBA initiation and TPA promotion protocol *(22)*. Megosh et al. also generated ODC-overexpressing transgenic mice *(23)*, but in these transgenic mice, stable ODC was targeted to the hair follicle using bovine keratin 6 (K6) promoter. A stable ODC expression vector was generated by deleting the PEST sequences from ODC complimentary DNA. As is discussed in Subheading 3, ODC is a labile protein that has a half-life of only 10–20 min. Mammalian ODC is degraded by at least two different pathways comprising constitutive and polyamine-dependent pathways. The PEST sequences (proline [P], glutamic acid [E], serine [S], and threonine [T]) are actually considered as signal structures for rapid and selective degradation of ODC by the proteosome. These K6 ODC transgenic mice spontaneously developed skin papillomas and carcinomas after DMBA initiation or overexpression of v-Ha-ras. These

results indicate that increased ODC activity, as observed in TPA promotion, is sufficient to accomplish the promotion stage of carcinogenesis *(24)*. The enhanced skin tumor development in K6/ODC mice either by DMBA initiation or by photocarcinogenesis was prevented by DFMO *(16)*. These results indicated that increased ODC activity is the key molecule that sensitizes skin for the development of skin tumors.

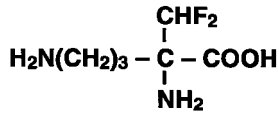
Further evidence implicating ODC and resultant polyamines in cancer formation emerged from a study involving manipulation of an ODC regulatory protein termed antizyme (AZ) *(25–28)*. AZ is a polyamine-induced cellular protein (molecular weight approx 23 kDa) involved in the feedback regulation of cellular polyamine levels. AZ binds directly to ODC and targets it to rapid ubiquitin-independent degradation by the 26S proteosome. AZ acts on ODC to enhance the association of ODC with proteosome and not the rate of this processing.

There are three isoforms of AZ. ODC AZs 1 and 2 are expressed ubiquitously, and the third, the testicular isoform AZ3, is expressed in differentiated haploid germ cells. AZ1, but not AZ2, accelerates ODC degradation. AZ1 binds ODC with about a three-fold higher potency that AZ2, but cannot account for their distinct degradation activities. Evidence also indicates that AZ is not only involved in ODC degradation, but in the negative regulation of polyamine transport. AZ activity is inhibited by the AZ inhibitor, which binds to the AZ with higher affinity than that of ODC, releasing ODC from the ODC–AZ complex *(27)*. AZ is a potential target to regulate cellular polyamine levels and prevent carcinogenesis. AZ overexpression in transgenic mice has been shown to reduce *N*-nitromethylbenzylamine-induced forestomach carcinogenesis *(26)*. Overexpression of ODC accelerated, but overexpression of AZ inhibited, induction of basal cell carcinogenesis by UVB in PtCh1$^{+/-}$ mice *(5)*.

Further evidence of the role of increase putrescine in skin tumor development was provided using transgenic mice overexpressing spermidine/spermine N^1-acetyltransferase (SSAT). SSAT is characterized by its inducibility and rapid turnover rate, and is the rate-limiting enzyme controlling conversion of spermidine and spermine to putrescine *(7)*. Colemen et al. generated SSAT-overexpressing transgenic mice (K6-SSAT) using the bovine K6 promoter. K6-SSAT transgenic mice appeared to be phenotypically normal and developed 10-fold more skin tumors as compared with wild-type mice in response to single DMBA initiation followed by twice weekly application of TPA. Tumor samples from transgenic mice showed elevated levels of SSAT activity and SSAT protein. These results lend support to the conclusion that activation of polyamine catabolism leading to increases in putrescine and N^1-acetylspermidine may be linked to chemically induced mouse skin neoplasia *(7)*. In contrast, Pietila et al. have reported that SSAT overexpressing transgenic mice developed significantly fewer papillomas than their syngenic littermates *(29)*.

3. ODC Regulation

Molecules that signal ODC induction may be possible targets for cancer prevention and treatment. TPA-induced ODC activity is regulated at least in part at the transcriptional level mediated by non-AP-1 transcriptional factors *(30–32)*. In this context, the link of c-Myc in the transcriptional regulation of ODC is noteworthy. c-Myc, an oncogenic transcription factor, is involved in the regulation of various cellular functions. ODC



α – DIFLUOROMETHYLORNITHINE

Fig. 4. Structure of DFMO, a suicide inhibitor of ornithine decarboxylase.

is a transcriptional target for c-Myc. *ODC* gene has two conserved functional CACGTG-c-myc binding sites in the first intron, the CATGTG motif in the 5¢-flanking region. Transactivation of the ODC promoter also requires the dimerization of c-Myc and Max.

3.1. Protein Kinase C, ODC Induction, and SCC

We also determined the initial signal(s) that are linked to ODC induction by TPA. TPA binds specifically with high affinity to its receptor(s) identified as protein kinase C (PKC) *(33–35)*. PKC, which is ubiquitous in eukaryotes, is a major intercellular receptor for the mouse skin tumor promoter, TPA. PKC forms part of the signal transduction system involving the turnover of inositol phospholipids and is activated by DAG, which is produced as a consequence of this turnover. On the basis of the structural similarities and cofactor requirements, the PKC isoforms have been grouped into three subfamilies of enzymes (Fig. 5).

The conventional PKCs (a, bI, bII, and g), which are dependent on DAG/TPA, Ca^{2+}, and phosphatidylserine (PS); the novel PKCs (d, e, q, and h), which require only PS and DAG/TPA; and the atypical PKCs (z, l, and i), which retain only the PS dependence but have no requirement for DAG/TPA or Ca^{2+} for activation (PKCm, which is usually classified as an nPKC, is not easily grouped with any of the other isoforms) *(36–38)*. At least six PKC isoforms (a, d, e, h, z, and m) are expressed in mouse skin. To determine the in vivo functional specificity of PKCa, -d, and -e in TPA-activated PKC signals to ODC induction and skin tumor multiplicity, we generated transgenic mice that expressed T7-epitope-tagged PKCa, -d, or -e in their epidermis. The expression of individual PKC isoforms was directed to the basal cells of the epidermis using a human cytokeratin 14 (K14) promoter *(33–35)*. Overexpression of PKCa did not affect the induction of skin tumors elicited by the initiation (DMBA)-promotion (TPA) protocol *(33,39)*. However, overexpression of PKCd suppressed the formation of both skin papillomas and carcinomas *(34)*, whereas PKCe transgenic mice developed papilloma-independent metastatic squamous cell carcinomas (Fig. 6) *(35,40)*. Interestingly, despite the different skin tumor susceptibilities of PKCa, PKCd, and PKCe transgenic mice, PKCd and PKCe, but not PKCa, overexpressing transgenic mice superinduced epidermal ODC activity compared with wild-type littermates after TPA treatment *(33)*.

3.2. PKCd-Induced ODC Activity is not Linked to the Induction of Skin

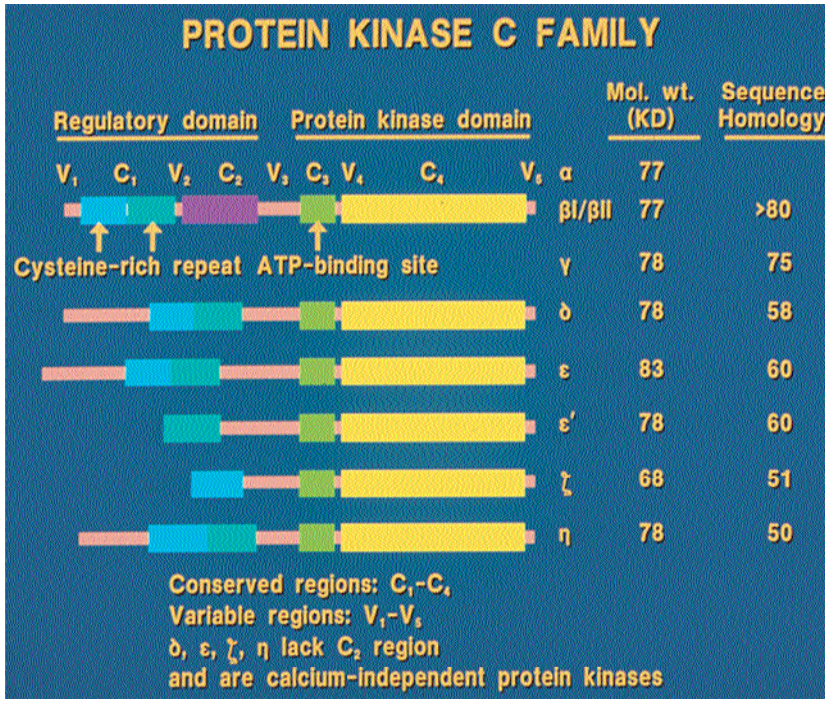


Fig. 5. Protein kinase C family.

Tumors

PKCδ occupies a central position in a cell's signaling network that regulates a wide variety of cellular processes, which include suppression of cell proliferation and transformation, and induction of apoptosis and differentiation *(34)*. We found PKCδ to be a potent suppressor of the induction of both papillomas and carcinomas in intact mouse skin in vivo. The mechanism by w hich PKCδ overexpression results in inhibition of skin tumor formation is yet unclear. TPA-induced ODC activity has been shown to be correlated with mouse skin tumor promotion *(24)*. Such a correlation was not observed in PKCδ transgenic mice. On the contrary, PKCδ overexpression suppressed TPA-induced tumor formation, whereas it superinduced ODC activity, indicating that PKCδ-mediated signals to ODC induction and skin tumor suppression are not on the same linear pathway. TPA-induced *ODC* gene expression appears to be mediated by PKCδ activation. Specific examples are the observations that co-transfection of a PKCδ expression vector with an ODC promoter (772/+130 ODC-Luc) enhanced the reporter gene activity *(41)* and inhibition of PKCδ activity by use of a dominant–negative PKCδ inhibited the induction of ODC activity *(42)*. The transgenic mice, which overexpress PKCδ in their epidermis, are more responsive to TPA for the induction of ODC activity than their wild-type littermates. Overinduction of ODC was observed either after a single or repeated TPA application to skin. It is noteworthy that the levels of both epi-

dermal PKCδ protein and PKCδ activity remained increased, which correlated with the induction of ODC activity in PKCδ transgenic mice. It appears that the PKCδ activity level determines the degree of induction of ODC activity by TPA. DFMO, which inhibits TPA-induced ODC activity *(43)*, did not alter the response of PKCδ transgenic mice to skin tumor promotion by TPA. If ODC had been downstream of PKCδ signals to skin tumor suppression, then inhibition of ODC activity by DFMO treatment could have reversed the tumor susceptibility of PKCδ transgenic mice to their wild-type littermates. The lack of effect of DFMO treatment on the sensitivity of PKCδ transgenic mice to skin tumor promotion by TPA indicates that the PKCδ-mediated signals to ODC induction and skin tumor suppression are not on the same linear pathway. The lack of correlation between TPA-induced ODC activity and skin tumor multiplicity in PKCδ transgenic mice can be explained as follows: ODC is the key enzyme in the biosynthesis of mammalian polyamines, which regulate a wide variety of cellular processes. The level of polyamines is regulated at the levels of biosynthesis, interconversion, and degradation *(44,45)*. Polyamines spermidine and spermine are acetylated and then excreted to maintain the intracellular polyamine pool. Although ODC is superinduced in PKCδ transgenic mice, it is likely that because of interconversion, degradation, and excretion that a critical level of polyamines or spermidine/spermine ratio essential to favor tumor promotion is never achieved. PKCδ may activate a signaling pathway, independent of one leading to ODC induction, which may interfere with polyamine specific signals to skin tumor promotion by TPA *(46)*.

3.3. PKCε Activation Linked to ODC Induction and SCC

The role of TPA-stimulated polyamine biosynthesis in the development of metastatic squamous cell carcinoma (mSCC) in PKCε transgenic mice was determined. TPA treatment induced epidermal ODC activity and putrescine levels approx three- to fourfold more in PKCε transgenic mice than their wild-type littermates. Development of mSCC by the 7,12-dimethylbenz(*a*)anthracene (100 nmol)-TPA (5 nmol) protocol in PKCε transgenic mice was completely prevented by administration of the suicide inhibitor of ODC-DFMO (0.5% w/v) in the drinking water during TPA promotion. However, DFMO treatment led to marked hair loss in PKCε transgenic mice. DFMO treatment-associated hair loss in PKCε transgenic mice was accompanied by a decrease in the number of intact hair follicles. These results indicate that TPA-induced ODC activity and the resultant accumulation of putrescine in PKCε transgenic mice are linked to growth and maintenance of hair follicles and the development of mSCC *(47)*.

When the FVB/N wild-type mice were given 0.5% DFMO in their drinking water during tumor promotion, little effect in hair loss was noted. However, PKCε transgenic mice exhibited severe hair loss, which on histological examination revealed almost complete loss of hair follicles. These results indicate that PKCε and ODC may play, in concert, pivotal roles in the development, growth, and maintenance of the hair follicle. ODC expression has been linked to hair follicle cycling. ODC is abundantly expressed in the lower part of the follicular bulb in the anagen (growth phase) of the hair cycle, whereas no expression is detected in either the catagen (regression phase) or telogen

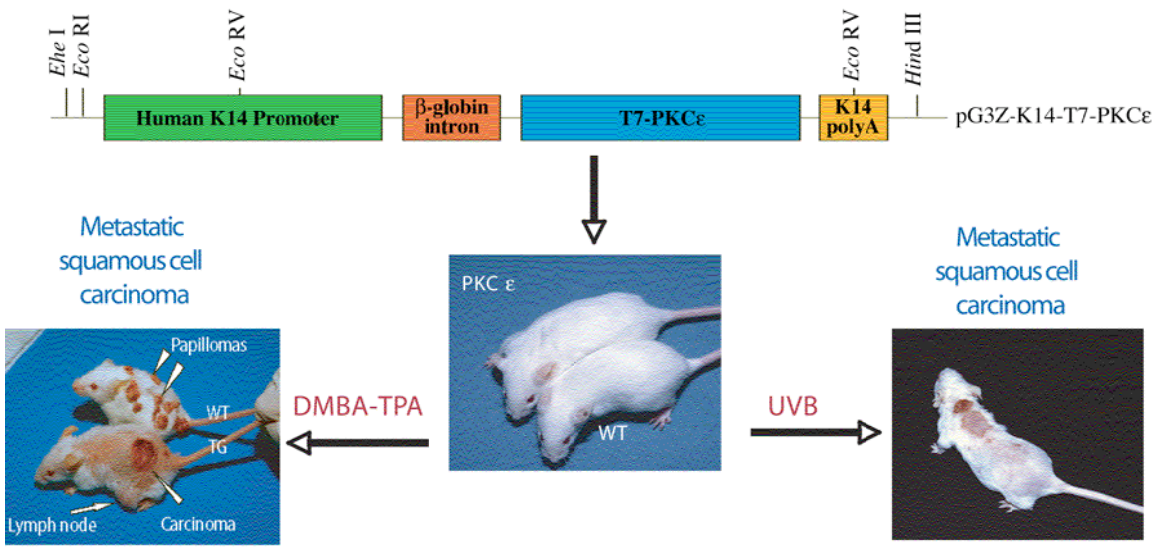


Fig. 6. Protein kinase Cε overexpression sensitizes skin through the induction of squamous cell carcinoma, either by the DMBA-TPA protocol or repeated ultraviolet radiation exposure.

(resting phase) phases of the hair cycle. As compared with the wild-type littermates, PKC transgenic mice exhibited hyperplasia of the hair follicle during skin tumor promotion by TPA. DFMO treatment, in conjunction with TPA promotion, led to almost complete loss of the hair follicle with an extreme thickening of the interfollicular epidermis in PKCε transgenic mice. The target cells for skin papillomas and carcinomas have been postulated previously either to be the basal keratinocytes of the interfollicular epidermis or the cells that form the outer root sheath of the hair follicle. The results presented here additionally strengthen our findings that the carcinomas in PKCε transgenic mice originate from cells in the hair follicle *(47)*.

3.3.1. TNF-α Linked to PKCε-Mediated Development of SCC

We found that PKCε transgenic mice elicit elevated serum TNF-α levels during skin tumor promotion by TPA, and this increase may be linked to the development of mSCC. A single topical application of TPA (5 nmol) to the skin, as early as 2.5 h after treatment, resulted in a significant ($p < 0.01$) increase (twofold) in epidermal TNF-α, and more than a sixfold increase in ectodomain shedding of TNF-α into the serum of PKCε transgenic mice relative to their wild-type littermates. Furthermore, this TPA-stimulated TNF-α shedding was proportional to the level of expression of PKCε in the epidermis. Using the TNF-α-converting enzyme (TACE) inhibitor, TAPI-1, TPA-stimulated TNF-α shedding could be completely prevented in PKCε transgenic mice and isolated keratinocytes. These results indicate that PKCε signal transduction pathways to TPA-stimulated TNF-α ectodomain shedding are mediated by TACE, a transmembrane metalloprotease. Using the superoxide dismutase mimetic CuDIPs and the glutathione reductase mimetic ebselen, TPA-stimulated TNF-α shedding from PKCε transgenic mice could be completely attenuated, implying the role of reactive oxygen species. Finally, ip injection of a TNF-α synthesis inhibitor, pentoxifylline, during skin tumor promotion completely prevented the development of mSCC in PKCε transgenic mice. Taken together, these results indicate that: PKCε activation is an initial signal in TPA-induced shedding of TNF-α from epidermal keratinocytes; PKCε-mediated signals to TACE are possibly mediated through reactive oxygen species; and TPA-induced TNF-α shedding may play a role in the development of mSCC in PKCε transgenic mice *(48)*.

The role of TNF-α in UVR-induced cutaneous damage was also evaluated using PKCε transgenic mice deficient in TNF-α *(13)*. UVR treatment, three times weekly for 13 wk at 2 kJ/m^2, induced severe cutaneous damage in PKCε transgenic mice (line 215), which was prevented in PKCε transgenic–TNF-α knockout mice. Taken together, the results indicate that PKCε signals UVR-induced TNF-α release that is linked, at least in part, to the photosensitivity of PKCε transgenic mice.

PKCε transgenic mice were also observed to be more sensitive than their wild-type littermates to UVR-induced release of cytokines (interleukin [IL]-5, IL-6, IL-10; granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor) other than TNF-α. It is likely that there is crosstalk among cytokines. It is notable that TNF-α has been shown to regulate the production of several cytokines.

TNF-α signal transduction pathways in UVR-induced cutaneous damage and development of SCC are not known. TNF-α mediates the activation of two transcriptional

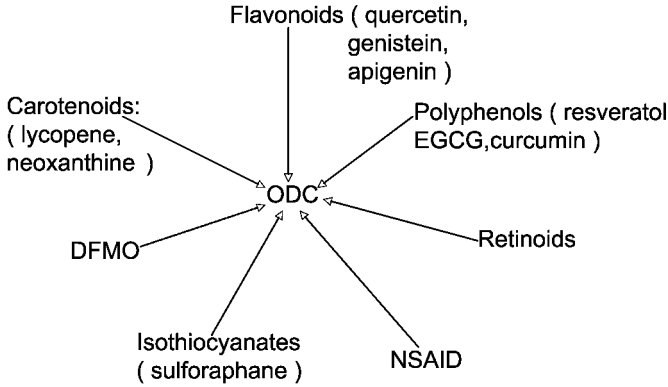


Fig. 7. Ornithine decarboxylase is a potential surrogate endpoint biomarker for human cancer prevention trials.

factors, AP-1 and nuclear factor (NF)-kB, linked to the expression of TNF-a-induced genes involved in immunity and inflammatory responses, and control of cellular proliferation, differentiation, and apoptosis *(49,50)*. The role of AP-1 and NF-kB activation in TNF-a signal transduction pathway to the development of SCC and PKCe transgenic mice is unknown and is important in view of the fact that NF-kB activation is an oncogenic signal in systems other than skin *(49,50)*.

An analysis of TPA signal transduction pathways have led to the identification of various targets linked to ODC induction and SCC. These target molecules include: PKCe, cytokine TNF-a and granulocyte colony-stimulating factor, prostaglandins, free radicals, and transcriptional factor AP-1. Based on the knowledge of the signaling molecules of ODC induction by TPA, various specific inhibitors have been designed which has been shown to preclude ODC induction and subsequent accumulation of putrescine and induction of cancer in various organs. ODC is a potential surrogate biomarker for a number of chemopreventive agents being evaluated for human cancer prevention trials (Fig. 7).

4. Conclusions

Experimental evidence involving biochemical, pharmacological, and genetic approaches unequivocally supports that deregulation of polyamine biosynthesis and catabolism are the hallmarks of malignancy *(6–8)*. Data from animal models for human cancer implicated the potential of enzymes of polyamine biosynthesis and interconversion as molecular targets for cancer prevention and treatment *(6–8)*. ODC has been extensively explored for intervention of cancer formation *(1,3,6)*. DFMO, a suicide inhibitor of ODC, has never failed to inhibit the induction of cancer (e.g., skin, colon, prostate, mammary, intestine, pancreas, bladder, liver, cervical, stomach) in animals *(1)*. DFMO is currently being evaluated in human cancer prevention trials in populations at high risk for cancers of the skin, prostate, colon, breast, cervix, and esophagus *(1)*. DFMO as a single agent may not be effective at the desired nontoxic dose. Also, toxicity associated with polyamine analogs hinders their use in prevention and treatment of cancer *(51–56)*. The

use of a combination of agents may be a better approach in the prevention of cancer. A combination of inhibitors with different molecular targets may show enhanced efficacy at levels below the threshold for undesirable side effects of each when used singly.

References

1. Gerner, E. W. and Meyskens, F L., Jr. (2004) Polyamines and cancer: Old molecules, new understanding. *Nat. Rev. Cancer* **4,** 781–792.
2. Wallace, H. M., Fraser, A. V., and Hughes, A. (2003) A perspective of polyamine metabolism. *Biochem. J.* **376,** 1–14.
3. Thomas, T. and Thomas, T. J. (2003) Polyamine metabolism and cancer. *J. Cell Mol. Med.* **7,** 113–126.
4. Scalabrino, G. and Ferioli, M. E. (1985) Degree of enhancement of polyamine biosynthetic decarboxylase activities in human tumors: A useful new index of degree of malignancy. *Cancer Detect. Prev.* **8,** 11–16.
5. Tang, X., Kim, A. L., Feith, D. J., et al. (2004) Ornithine decarboxylase is a target for chemoprevention of basal and squamous cell carcinomas in Ptch1+/- mice. *J. Clin. Invest.* **113,** 867–875.
6. Pegg, A. E., Feith, D. J., Fong, L. Y., Coleman, C. S., O'Brien, T. G., and Shantz, L. M. (2003) Transgenic mouse models for studies of the role of polyamines in normal, hypertrophic and neoplastic growth. *Biochem. Soc. Trans.* **31,** 356–360.
7. Coleman, C. S., Pegg, A. E., Megosh, L. C., Guo, Y., Sawicki, J. A., and O'Brien, T. G. (2002) Targeted expression of spermidine/spermine N1-acetyltransferase increases susceptibility to chemically induced skin carcinogenesis. *Carcinogenesis* **23,** 359–364.
8. Feith, D. J., Shantz, L. M., and Pegg, A. E. (2001) Targeted antizyme expression in the skin of transgenic mice reduces tumor promoter induction of ornithine decarboxylase and decreases sensitivity to chemical carcinogenesis. *Cancer Res.* **61,** 6073–6081.
9. DiGiovanni, J. (1992) Multistage carcinogenesis in mouse skin. *Pharmacol. Ther.* **54,** 63–128.
10. Yuspa, S. H., Dlugosz, A. A., Denning, M. F., and Glick, A. B. (1996) Multistage carcinogenesis in the skin. *J. Invest. Dermatol. Symp. Proc.* **1,** 147–150.
11. Mukhtar, H., Forbes, P. D., and Ananthaswamy, H. N. (1999) Photocarcinogenesis—models and mechanisms. *Photodermatol. Photoimmunol. Photomed.* **15,** 91–95.
12. de Gruijl, F. R. (1999) Skin cancer and solar UV radiation. *Eur J. Cancer* **35,** 2003–2009.
13. Wheeler, D. L., Martin, K. E., Ness, K. J., et al. (2004) Protein kinase C epsilon is an endogenous photosensitizer that enhance ultraviolet radiation-induce cutaneous damage and development of squamous cell carcinomas. *Cancer Res.* **64,** 7756–7765.
14. Fujiki, H., Suganuma, M., Sururi, H., et al. (1989) New tumor promoters from marine sources: The okadaic acid class. In: *Mycotoxins and Phycotoxins '88: A Collection of Invited Papers Presented at the Seventh International IUPAC Symposium on Mycotoxins and Phycotoxins, Tokyo, Japan, 16–19 August 1988* (Natori, S., Hasimoto, K., and Ueno, Y., eds.), Elsevier, Amsterdam, p. ix.
15. Arbeit, J. M., Riley, R. R., Huey, B., et al. (1999) Difluoromethylornithine chemoprevention of epidermal carcinogenesis in K14-HPV16 transgenic mice. *Cancer Res.* **59,** 3610–3620.
16. Gupta, S., Ahmad, N., Marengo, S. R., MacLennan, G. T., Greenberg, N. M., and Mukhtar, H. (2000) Chemoprevention of prostate carcinogenesis by alpha-difluoromethylornithine in TRAMP mice. *Cancer Res.* **60,** 5125–5133.

17. Lao, C. D., Backoff, P., Shotland, L. I., et al. (2004) Irreversible ototoxicity associated with difluoromethylornithine. *Cancer Epidemiol. Biomarkers Prev.* **13,** 1250–1252.
18. Verma, A. K. (1990) Inhibition of tumor promotion by DL-alpha-difluoromethylornithine, a specific irreversible inhibitor of ornithine decarboxylase. *Basic Life Sci.* **52,** 195–204.
19. Fischer, S. M., Conti, C. J., Viner, J., Aldaz, C. M., and Lubet, R. A. (2003) Celecoxib and difluoromethylornithine in combination have strong therapeutic activity against UV-induced skin tumors in mice. *Carcinogenesis* **24,** 945–952.
20. Wattenberg, L. W., Wiedmann, T. S., and Estensen, R. D. (2004) Chemoprevention of cancer of the upper respiratory tract of the Syrian golden hamster by aerosol administration of difluoromethylornithine and 5-fluorouracil. *Cancer Res.* **64,** 2347–2349.
21. Alberts, D. S., Dorr, R. T., Einspahr, J. G., et al. (2000) Chemoprevention of human actinic keratoses by topical 2-(difluoromethyl)-dl-ornithine. *Cancer Epidemiol. Biomarkers Prev.* **9,** 1281–1286.
22. Halmekyto, M., Syrjanen, K., Janne, J., and Alhonen, L. (1992) Enhanced papilloma formation in response to skin tumor promotion in transgenic mice overexpressing the human ornithine decarboxylase gene. *Biochem. Biophys Res. Commun.* **187,** 493–497.
23. Megosh, L., Gilmour, S. K., Rosson, D., et al. (1995) Increased frequency of spontaneous skin tumors in transgenic mice which overexpress ornithine decarboxylase. *Cancer Res.* **55,** 4205–4209.
24. O'Brien, T. G., Megosh, L. C., Gilliard, G., and Soler, A. P. (1997) Ornithine decarboxylase overexpression is a sufficient condition for tumor promotion in mouse skin. *Cancer Res.* **57,** 2630–2637.
25. Heller, J. S. and Canellakis, E. S. (1981) Cellular control of ornithine decarboxylase activity by its antizyme. *J. Cell Physiol.* **107,** 209–217.
26. Fong, L. Y., Feith, D. J., and Pegg, A. E. (2003) Antizyme overexpression in transgenic mice reduces cell proliferation, increases apoptosis, and reduces N-nitrosomethylbenzylamine-induced forestomach carcinogenesis. *Cancer Res.* **63,** 3945–3954.
27. Zhang, M., Pickart, C. M., and Coffino, P. (2003) Determinants of proteasome recognition of ornithine decarboxylase, a ubiquitin-independent substrate. *EMBO J.* **22,** 1488–1496.
28. Nilsson, J., Grahn, B., and Heby, O. (2000) Antizyme inhibitor is rapidly induced in growth-stimulated mouse fibroblasts and releases ornithine decarboxylase from antizyme suppression. *Biochem. J.* **346,** 699–704.
29. Pietila, M., Pirinen, E., Keskitalo, S., et al. (2005) Disturbed keratinocyte differentiation in transgenic mice and organotypic keratinocyte cultures as a result of spermidine/spermine N-acetyltransferase overexpression. *J. Invest. Dermatol.* **124,** 596–601.
30. Reddig, P. J., Kim, Y. J., and Verma, A. K. (1996) Localization of the 12-O-tetradecanoylphorbol-13-acetate response of the human ornithine decarboxylase promoter to the TATA box. *Mol. Carcinogen* **17,** 92–104.
31. Kennard, M. D., Kang, D. C., Montgomery, R. L., and Butler, A. P. (1995) Expression of epidermal ornithine decarboxylase and nuclear proto-oncogenes in phorbol ester tumor promotion-sensitive and -resistant mice. *Mol. Carcinogen* **12,** 14–22.
32. Auvinen, M., Jarvinen, K., Hotti, A., et al. (2003) Transcriptional regulation of the ornithine decarboxylase gene by c-Myc/Max/Mad network and retinoblastoma protein interacting with c-Myc. *Int. J. Biochem. Cell Biol.* **35,** 496–521.
33. Jansen, A. P., Dreckschmidt, N. E., Verwiebe, E. G., Wheeler, D. L., Oberley, T. D., and Verma, A. K. (2001) Relation of the induction of epidermal ornithine decarboxylase and hyperplasia to the different skin tumor-promotion susceptibilities of protein kinase C-alpha, -delta and -epsilon transgenic mice. *Int. J. Cancer* **93,** 635–643.

34. Reddig, P. J., Dreckschmidt, N. E., Ahrens, H., et al. (1999) Transgenic mice overexpressing protein kinase Cdelta in the epidermis are resistant to skin tumor promotion by 12-O-tetradecanoylphorbol-13-acetate. *Cancer Res.* **59,** 5710–5718.
35. Reddig, P. J., Dreckschmidt, N. E., Zou, J., Bourguignon, S. E., Oberley, T. D., and Verma, A. K. (2000) Transgenic mice overexpressing protein kinase C epsilon in their epidermis exhibit reduced papilloma burden but enhanced carcinoma formation after tumor promotion. *Cancer Res.* **60,** 595–602.
36. Mellor, H. and Parker, P. J. (1998) The extended protein kinase C superfamily. *Biochem. J.* **332,** 281–292.
37. Mochly-Rosen, D. and Kauvar, L. M. (1998) Modulating protein kinase C signal transduction. *Adv. Pharmacol.* **44,** 91–145.
38. Newton, A. C. (1995) Protein kinase C: Structure, function, and regulation. *J. Biol. Chem.* **270,** 28,495–28,498.
39. Wang, H. Q., and Smart, R. C. (1999) Overexpression of protein kinase C-alpha in the epidermis of transgenic mice results in striking alterations in phorbol ester-induced inflammation and COX-2, MIP-2 and TNF-alpha expression but not tumor promotion. *J. Cell Sci.* **112,** 3497–3506.
40. Jansen, A. P., Verwiebe, E. G., Dreckschmidt, N. E., Wheeler, D. L., Oberley, T. D., and Verma, A. K. (2001) Protein kinase C-epsilon transgenic mice: a unique model for metastatic squamous cell carcinoma. *Cancer Res.* **61,** 808–812.
41. Tseng, C. P., Kim, Y. J., Kumar, R., and Verma, A. K. (1994) Involvement of protein kinase C in the transcriptional regulation of 12-O-tetradecanoylphorbol-13-acetate-inducible genes modulated by AP-1 or non-AP-1 transacting factors. *Carcinogenesis* **15,** 707–711.
42. Otieno, M. A. and Kensler, T. W. (2000) A role for protein kinase C-delta in the regulation of ornithine decarboxylase expression by oxidative stress. *Cancer Res.* **60,** 4391–4396.
43. Verma, A. K. (1989) The enzyme-activated irreversible inhibitor of ornithine decarboxylase, DL-alpha-difluoromethylornithine: A chemopreventive agent. *Prev. Med.* **18,** 646–652.
44. Pegg, A. E. (1986) Recent advances in the biochemistry of polyamines in eukaryotes. *Biochem. J.* **234,** 249–262.
45. Igarashi, K. and Kashiwagi, K. (2000) Polyamines: Mysterious modulators of cellular functions. *Biochem. Biophys Res. Commun.* **271,** 559–564.
46. Wheeler, D. L., Reddig, P. J., Dreckschmidt, N. E., Leitges, M., and Verma, A. K. (2002) Protein kinase Cdelta-mediated signal to ornithine decarboxylase induction is independent of skin tumor suppression. *Oncogene* **21,** 3620–3630.
47. Wheeler, D. L., Ness, K. J., Oberley, T. D., and Verma, A. K. (2003) Inhibition of the development of metastatic squamous cell carcinoma in protein kinase C epsilon transgenic mice by alpha-difluoromethylornithine accompanied by marked hair follicle degeneration and hair loss. *Cancer Res.* **63,** 3037–3042.
48. Wheeler, D. L., Ness, K. J., Oberley, T. D., and Verma, A. K. (2003) Protein kinase Cepsilon is linked to 12-O-tetradecanoylphorbol-13-acetate-induced tumor necrosis factor-alpha ectodomain shedding and the development of metastatic squamous cell carcinoma in protein kinase Cepsilon transgenic mice. *Cancer Res.* **63,** 6547–6555.
49. Baud, V. and Karin, M. (2001) Signal transduction by tumor necrosis factor and its relatives. *Trends Cell Biol.* **11,** 372–377.
50. Karin, M., Cao, Y., Greten, F. R., and Li, Z. W. (2002) NF-kappaB in cancer: From innocent bystander to major culprit. *Nat. Rev. Cancer* **2,** 301–310.

51. Kong Thoo Lin, P., Dance, A. M., Bestwick, C., and Milne, L. (2003) The biological activities of new polyamine derivatives as potential therapeutic agents. *Biochem. Soc. Trans.* **31,** 407–410.
52. Thomas, T., Balabhadrapathruni, S., Gallo, M. A., and Thomas, T. J. (2002) Development of polyamine analogs as cancer therapeutic agents. *Oncol. Res.* **13,** 123–135.
53. Marton, L. J. and Pegg, A. E. (1995) Polyamines as targets for therapeutic intervention. *Annu. Rev. Pharmacol. Toxicol.* **35,** 55–91.
54. Denstman, S. C., Ervin, S. J., and Casero, R. A., Jr. (1987) Comparison of the effects of treatment with the polyamine analogue N1,N8 bis(ethyl)spermidine (BESpd) or difluoromethylornithine (DFMO) on the Topoisomerase II mediated formation of 4¢-(9-acridinylamino) methanesulfon-m-anisidide (m-AMSA) induced cleavable complex in the human lung carcinoma line NCI H157. *Biochem. Biophys Res. Commun.* **149,** 194–202.
55. Casero, R. A., Jr., Go, B., Theiss, H. W., Smith, J., Baylin, S. B., and Luk, G. D. (1987) Cytotoxic response of the relatively difluoromethylornithine-resistant human lung tumor cell line NCI H157 to the polyamine analogue N1,N8-bis(ethyl)spermidine. *Cancer Res.* **47,** 3964–3967.
56. Casero, R. A., Jr., Bergeron, R. J., and Porter, C. W. (1984) Treatment with alpha-difluoromethylornithine plus a spermidine analog leads to spermine depletion and growth inhibition in cultured L1210 leukemia cells. *J. Cell Physiol.* **121,** 476–482.

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Polyamine Pools and Cancer Prevention

Eugene W. Gerner and David E. Stringer

1. Introduction

Polyamine contents increase during epithelial carcinogenesis in both rodents and humans. The activity of ornithine decarboxylase (ODC), the first enzyme in polyamine synthesis in mammals, is increased in epithelial cancers, and ODC has become a target for cancer chemoprevention. Difluoromethylornithine (DFMO) is a specific inhibitor of ODC and is under evaluation in clinical trials for prevention of epithelial cancers, including those of the colon, prostate, and skin. Tissue, serum, and other bodily fluid endpoints have been used to monitor effects of DFMO in human clinical chemoprevention trials. Cell and tissue polyamine contents are regulated by mechanisms influencing synthesis, catabolism, uptake, and efflux. These regulatory mechanisms include both transcriptional and posttranscriptional processes. Tissue measurements of ODC enzyme activity have often failed to be reliable measures of either carcinogenesis or response to DFMO. Levels of target tissue polyamines are less subject to measurement variability than are measures of ODC enzyme activity, and are closely associated with administered DFMO dose in randomized clinical chemoprevention trials in humans. Target tissue polyamine contents are validated biomarkers for the biochemical efficacy of DFMO in clinical cancer chemoprevention trials. There is a need for new predictive biomarkers of agents that affect polyamine metabolism that could be measured in sources other than target tissues.

2. Regulation of Polyamine Metabolic Genes in Normal Development

Polyamine metabolic genes are targets of several signaling pathways regulating normal development in mammals. ODC, the first enzyme in polyamine synthesis in animals, is a transcriptional target of the *c-MYC* gene *(1)*. The c-MYC protein is a transcriptional regulator of survival and apoptotic responses *(2)*, and is regulated in part by the adenomatous polyposis coli (*APC*) tumor suppressor gene *(3)*. *APC* regulates *ODC* transcription in intestinal and colonic tissues of mice *(4)*. *APC* regulation of *ODC* transcription occurs via a c-MYC-dependent mechanism in human colon tumor cells *(5)*. Wnt and Hedgehog are two important developmental regulatory pathways in

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eukaryotes that have also been associated with carcinogenesis *(6)*. Polyamine metabolic genes are targets of these pathways. The Wnt pathway member, APC, regulates ODC, whereas the ODC inhibitor protein antizyme has been reported to be a target of the Hedgehog pathway *(7)*. ODC is essential for cell survival during embryonic development in mice *(8)*. ODC expression is downregulated via a posttranscriptional mechanism in senescing human fibroblasts *(9)*, and putrescine and spermidine pools are lowest in slowly turning over adult epithelial tissues *(10)*. Reactivation of polyamine synthesis is crucial for repair processes in certain normal tissues *(11)*.

3. Regulation of Polyamine Metabolic Genes in Neoplasia

In humans suffering from familial adenomatous polyposis (FAP), an inherited form of colon cancer caused by loss of wild-type alleles encoding the *APC* gene, c-MYC, and subsequently ODC are aberrantly expressed *(12,13)*. Studies in the multiple intestinal neoplasia ($Apc^{Min/+}$) mouse model of FAP, and in genetically modified human cells, indicate that mutant APC is associated with an increase in ODC and a decrease in ODC inhibitor protein antizyme *(4)*. These changes in polyamine metabolic genes are associated with increases in intestinal mucosal polyamine contents in the $Apc^{Min/+}$ mice compared with normal littermates. Treatment of the $Apc^{Min/+}$ mice with the selective ODC inhibitor DFMO suppresses these elevated intestinal mucosal polyamine contents and reduces the number of intestinal adenomas. Other genes mutated or deleted in cancer also regulate polyamine metabolic genes. Activating mutations in the *K-RAS* oncogene, a signaling molecule activated by guanosine 5´-triphosphate, are found in about half of colon tumors induced by treatment of mice with the chemical carcinogen azoxymethane *(14)*. *K-RAS* activation is associated with the transcriptional repression of the spermidine/spermine N^1-acetyltransferase (SSAT), a regulatory enzyme in polyamine catabolism *(15)*. This transcriptional repression is mediated by the *K-RAS*-dependent suppression of expression of the peroxisomal proliferator-activated receptor gamma (PPARγ), which promotes SSAT transcription via a response element near the SSAT transcription start site *(16)*. Consequently, loss of function of the *APC* tumor suppressor and activation of the *K-RAS* oncogene, which are common events in human colon cancers, causes increases in intestinal polyamine contents because of increased synthesis and decreased catabolism of the polyamines. Evidence suggests that polyamine metabolism is altered in other epithelial cancers and neoplasia. Chemically induced skin carcinogenesis is associated with increases in ODC *(17)*, and ODC inhibitors are potent inhibitors of this process *(18)*. Polyamine metabolic gene expression is altered in human prostate cancer *(19)*. Recent studies indicate aberrant expression of c-MYC, the transcriptional activator of ODC in prostate epithelium, is sufficient to induce invasive prostate cancer in mice *(20)*. Treatment of the TRAMP mouse, another model of prostate cancer, with DFMO reduces prostate neoplasia (21). These studies provide evidence for causative roles of polyamine metabolic gene alterations in several types of epithelial cancer and neoplasia.

4. General Factors Controlling Polyamine Pool Sizes

The polyamines spermidine and spermine, and their diamine precursor, putrescine, derive from either intracellular synthesis, via decarboxylation of the amino acid

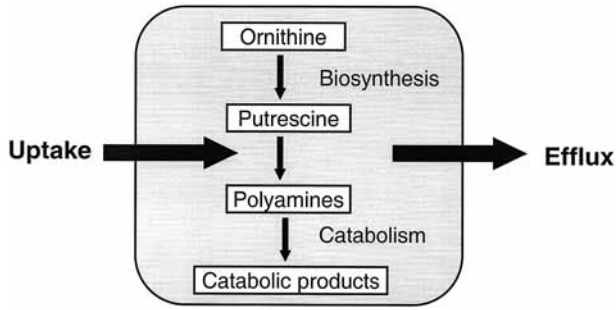

Fig. 1. In humans, increases in polyamine pools occur as a consequence of synthesis from ornithine or mechanisms transporting polyamines from extracellular sites into cells and tissues. Decreases in polyamine contents occur as a consequence of catabolism and export of intracellular pools to extracellular sites.

ornithine, or transport from extracellular sources in mammals. Figure 1 depicts these biosynthetic and uptake processes, along with catabolic and efflux mechanisms, all of which act to modulate intracellular polyamine pools.

These processes are highly regulated. Polyamines negatively regulate their synthesis and uptake from extracellular sources *(22)*, and positively regulate their catabolism and export *(23,24)*. Davis et al. *(25)* have noted a lack of evidence for polyamines allosterically regulating their own metabolism. They have argued that the apparent stability of polyamine pool sizes in unstressed cells is owing to sequestration of the polyamines, rather than as a consequence of allosteric feedback regulation. They present a compelling argument for the conclusion that only a small fraction of cellular polyamines are unbound, and consequently metabolically active. They point to observations that addition of exogenous polyamines to cultured mammalian cells can cause dramatic changes in intracellular processes involving polyamines, such as induction of the ODC antizyme, without causing detectable changes in intracellular polyamine pools. On the other hand, treatment of cultured mammalian cells with inhibitors of polyamine synthesis can deplete intracellular pools of some of the polyamines without dramatically affecting cell growth. These observations suggest that polyamine pools may be useful biomarkers to predict effects of agents that inhibit or activate polyamine metabolic enzymes, but may not be useful as markers of normal or disease processes.

5. Assessment of Errors in Measures of Polyamine Parameters in Human Tissues

Early in our cancer chemoprevention studies, it became clear that we needed to identify surrogate biomarkers as endpoints for our studies. We wanted to treat people who had not yet developed, but were at risk of developing, cancer. Development of colon and other epithelial cancers from noninvasive intraepithelial neoplasia has been estimated to take 15–30 yr. Thus, we determined sources of errors associated with several measures of polyamine parameters to determine which parameters were most reliably measured in human tissues. We conducted our most extensive study of errors in polyamine parameters

in colonic mucosa of normal volunteers *(26)*. We chose this particular tissue for study because it was relatively easily accessible and highly relevant to our planned studies of colon cancer chemoprevention. Our endpoints included measures of ODC and SSAT enzyme activities, *ODC* RNA expression, and polyamine contents. Enzyme activities were measured by documenting metabolism of radiolabeled substrates in tissue extracts, RNA expression was assessed by Northern blot analysis, and tissue polyamine contents were determined by high-performance liquid chromatography (HPLC) methods. Our measurement methods first used HPLC to separate individual amines, which were then derivatized with fluorescent markers. The fluorescently labeled amines were quantified. We evaluated measures of these endpoints in normal volunteers. First, we collected colonic tissues using several bowel preparation methods. We found that intraindividual measures of colorectal polyamine contents were less affected by bowel preparation methods compared with measures of enzyme activities. All measures of polyamine parameters in colorectal mucosa were highly variable between individuals. However, we found that differences in measures of polyamine contents, and especially spermidine:spermine ratios, were greatest between individuals. Differences in polyamine contents were less variable when compared in colorectal biopsies taken from similar locations in the same individual compared with those taken from different individuals. We evaluated variability in our measures of polyamine parameters in biopsies of various sizes taken at various locations in the colons of these normal volunteers. Finally, we evaluated measures of polyamine endpoints in biopsies obtained sequentially during insertion and extraction of the colonoscope to evaluate the influence of bowel location and irritation on these parameters. Measures of polyamine parameters were least variable when comparing values within individuals compared with those between individuals. Of all parameters measured, polyamine contents were least variable, especially spermidine:spermine ratios. We reasoned that the low intraindividual variability in spermidine:spermine ratios was a consequence of this parameter having only one source of technical measurement error. Both spermidine and spermine values are obtained from a single HPLC chromatogram, so that only technical measurement is required. Individual polyamine contents are determined as a ratio of two separate measurements, including the HPLC measurement, which is then normalized to tissue protein content. We concluded that measures of polyamine contents were the most reliable measures of polyamine parameters in colonic tissue of normal human volunteers. We reached the same conclusions regarding measurements of errors in polyamine parameters in other tissues in the intestinal tract *(10)* and prostate *(27)*, although our analysis of errors in these latter tissues was not as extensive as our initial studies in the colon.

6. Polyamine Parameters as Prognostic Markers in Cancer

Twenty yr ago, there was great interest in measures of polyamine metabolism as potential prognostic markers for epithelial cancers. Pioneering studies by Luk and Baylin found that ODC enzyme activity was elevated in the apparently normal colorectal mucosa of individuals with FAP compared with people not afflicted with FAP *(28)*. ODC enzyme activity and polyamine contents were found to be elevated in several examples of nonheritable intraepithelial neoplasia, including sporadic colon polyps

(29,30) and Barrett's esophagus *(31)*, compared with adjacent, normal tissues. Most of these studies found that average values of polyamine parameters were elevated in neoplasia compared with those in apparently normal tissue, but that substantial overlap existed between values in normal and neoplastic tissues. Further, measures of polyamine parameters in apparently normal colorectal mucosa were not prognostic for, or did not foretell the risk of, colon cancer development *(32–24)*. Measurements of polyamine parameters in human serum or urine were also found to be poor prognostic markers for cancer *(35)*, although recent technical developments, to be discussed at the end of this chapter, might provide new avenues for investigation. From these results, we concluded that ODC enzyme activity and polyamine contents were generally elevated in neoplastic compared with normal, intestinal tissue, but that measurements of these endpoints were not useful prognostic factors for people with either Barrett's esophagus or colon polyps.

7. Polyamine Parameters as Predictive Markers in Cancer Chemoprevention

Undeterred by these negative findings, we focused our attention on determining if polyamine parameters could be used as surrogate endpoint biomarkers in clinical cancer chemoprevention trials. The goal of cancer chemoprevention is to prevent the occurrence of invasive cancer. The target populations for chemoprevention trials are individuals at risk for, but who do not yet have, cancer. Because many epithelial cancers develop over one or more decades, clinical chemoprevention trials have used surrogate endpoints in trials lasting weeks to a few years to assess potential utility of agents for use in longer term trials *(36)*. When we initiated our cancer chemoprevention studies, DFMO had already been shown to be a potent inhibitor of carcinogenesis in several animal models *(37)*. Although a number of other inhibitors and activators of polyamine metabolism are now known, we have focused on DFMO because of its apparent safety when administered to humans *(37,38)*. Our choice of DFMO dose schedule merits discussion. Pharmacological studies of DFMO had shown that serum half-life of DFMO was on the order of hours in rodents, dogs, and humans *(39)*. These findings caused clinical investigators to employ multiple daily dosing or continuous intravenous infusion techniques to maintain maximal serum levels of DFMO in clinical trials. For cancer chemoprevention trials, we preferred simple methods of agent delivery. Cell culture studies with DFMO had shown that ODC enzyme activity continued to be suppressed, even after removal of DFMO from culture medium *(40)*. The persistent anti-ODC enzyme activity of DFMO was both DFMO concentration- and treatment time-dependent. We reasoned that DFMO was cleared rapidly from the serum, but that the ODC-inhibiting activity of the drug was longer lasting in cells and tissues. As a consequence of these published data and unpublished results, we chose an administration schedule of single daily oral doses of DFMO. Our early studies of DFMO involved drug administered orally in a liquid. We have recently changed to oral DFMO administered in tablet formulation.

8. Validation of Polyamine Contents as Biomarkers for DFMO Effect

Several polyamine parameters have been evaluated as potential biomarkers in chemoprevention trials of DFMO. Studies in rodent models showed that topical

application of 12-*O*-tetradecanoyl-13-acetate (TPA)-induced ODC enzyme activity in skin *(17)*. Uninduced ODC enzyme activity is difficult to detect in extracts of human skin punch biopsies. However, the University of Wisconsin group used these observations to evaluate inhibition of TPA-induced ODC activity in skin punch biopsies as a marker of DFMO effect in a chemoprevention trial *(41)*. They evaluated a range of doses to determine which oral DFMO doses, administered once daily, were able to inhibit ODC enzyme activity (induced by ex vivo TPA treatment) of punch biopsies by at least 50%. Their results indicated that doses of 0.5 g/m^2 per day could achieve this endpoint. Higher doses were found to cause reversible hearing loss, whereas lower doses failed to inhibit TPA-induced ODC activity by at least 50% in their ex vivo assay. Based on our evaluation of sources of errors in measurements of polyamine parameters, we chose to evaluate measures of polyamine contents as potential biomarkers of DFMO effect. In one study, we documented polyamine parameters in 111 men and women who had colon polyps. After removal of their polyps, we assessed polyamine contents, including spermidine:spermine ratios, in apparently normal rectal mucosa before beginning any drug treatment. In many experimental models, increased ODC synthesis is associated with increased putrescine and spermidine pool sizes, whereas spermine is often not affected. Thus, increases in spermidine:spermine ratios are general indicators of increased polyamine synthesis, whereas decreases in this parameter generally reflect decreases in polyamine synthesis *(40)*. As shown in Fig. 2, we found that spermidine:spermine ratios in rectal mucosa were negatively associated with age in a statistically significant manner.

This result was interesting because it was consistent with results discussed earlier, which showed that polyamine synthesis is downregulated in adult tissues and during senescence in experimental models. We conducted short-term trials of DFMO administration in humans with colon polyps. We wanted to identify the safest dose of DFMO to use in our chemoprevention trials because potential participants in these trials would be basically healthy individuals identified by risk factors for cancer. In collaboration with our long-time collaborator Frank Meyskens and his colleagues at the University of California at Irvine, we designed and conducted a unique dose-de-escalation trial of DFMO, with polyamine contents as our primary endpoint *(42)*. We found that DFMO taken as an oral liquid once per day for 1 mo reduced colorectal polyamine contents in a dose-dependent manner. In this nonrandomized trial, we found that DFMO dose was associated with decreased colorectal tissue contents of putrescine and spermidine, but not spermine. Spermidine:spermine ratios were also reduced in colorectal tissue in a statistically significant manner. Serum levels of DFMO were elevated in individuals taking this drug. Serum levels of DFMO were dependent on administered doses, but were highly variable among participants in the trial. Polyamine parameters, such as spermidine:spermine ratios, were reduced by administered dose, but not in a manner that was dependent on DFMO in the 0.1 to 3.0 g/m^2 range. As shown in Table 1, we found that single daily oral doses as low as 0.10 mg/m^2 or higher reduced spermidine:spermine ratios in the majority of patients at each dose group.

Patient responses measured by rectal spermidine:spermine ratios to oral DFMO were dependent on age, as seen in Fig. 3. These response data are consistent with the results

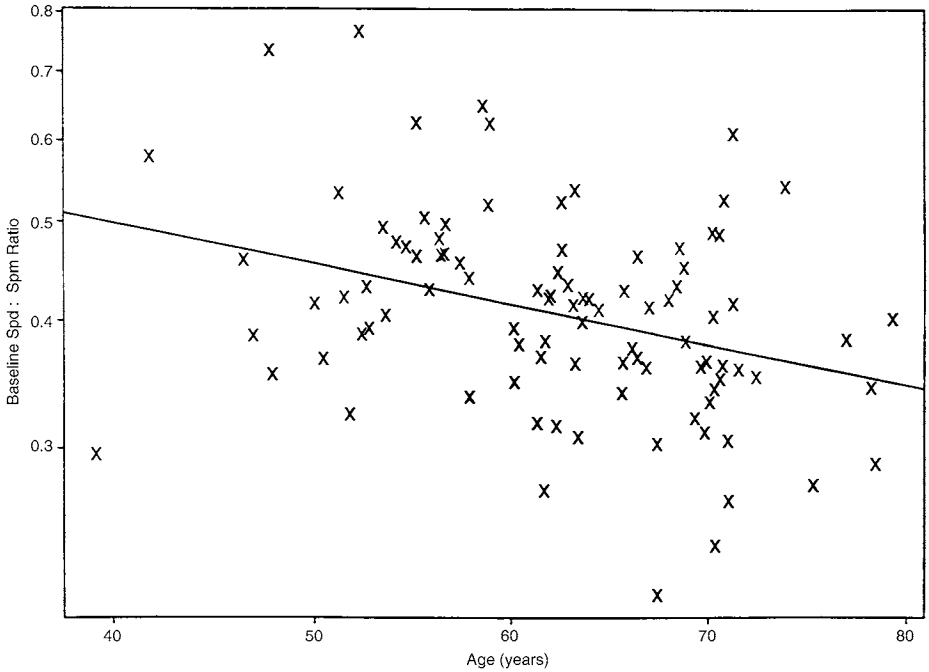

Fig. 2. Influence of patient age on spermidine:spermine ratios of colorectal mucosa. Biopsy specimens were obtained, with appropriate consents, from individuals who had a colon polyp, and were about to enroll in a chemoprevention trial of DFMO for colon polyp recurrence. Values shown are spermidine:spermine ratios before patients received DFMO. (Reprinted from ref. *42*.)

Table 1
Change in Colorectal Mucosal Spermidine:Spermine Ratios by DFMO Dose Group

DFMO dose (g/m^2/day)	Total number of participants	Number with decreased ratio	Number with increased ratio	*p* value
3.00	9	8	1	0.0391
1.50	12	11	1	0.0063
1.00	14	13	1	0.0018
0.75	16	14	2	0.0042
0.50	15	13	2	0.0074
0.25	15	13	2	0.0074
0.10	11	10	1	0.0117

Abstracted from ref. *42*.

shown in Fig. 2, and support the conclusion that ODC is downregulated as a function of age in apparently normal rectal mucosa. When ODC is downregulated with age, as seen by the decrease in spermidine:spermine ratios shown in Fig. 2, the ability of the ODC inhibitor DFMO to reduce spermidine:spermine ratios from baseline values also decreases.

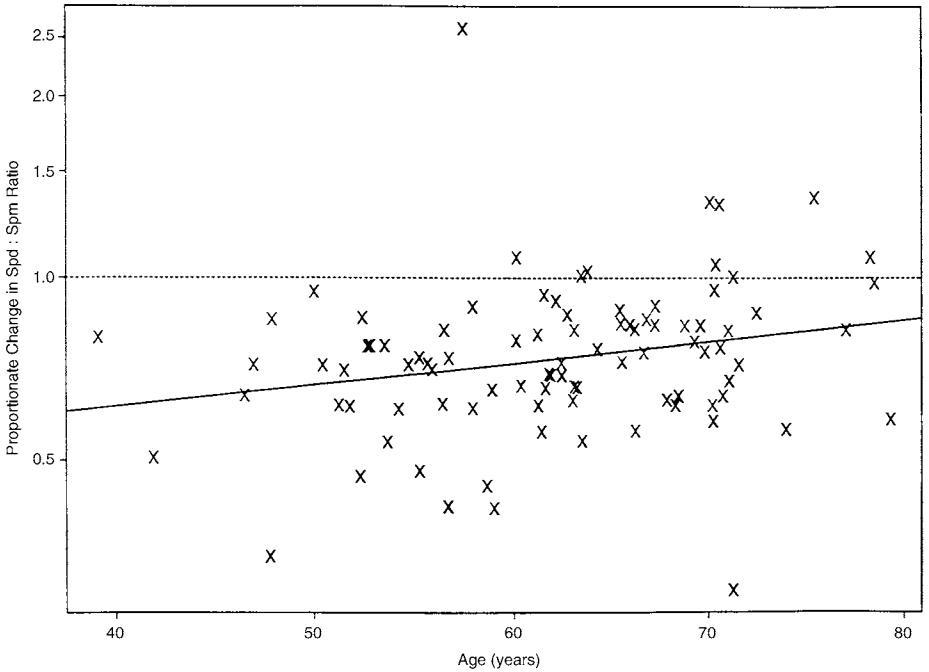

Fig. 3. Proportionate changes in ratios of spermidine:spermine in colorectal biopsies from patients, comparing values after 4 wk of DFMO treatment to baseline values, by participant age. (Reprinted from ref. *42*.)

Results from the de-escalation trial of DFMO doses for 1 mo were subsequently corroborated in a randomized clinical trial of 1-yr treatments with one of three DFMO doses vs placebo *(43)*. Eligibility for the trial included men and women, ages 40–80 yr, who had had an adenomatous polyp removed from their colon within the previous 5 yr before entering the trial. Patients were randomized to DFMO doses of 0.075, 0.20, and 0.40 g/m^2 or placebo. As shown in Fig. 4, putrescine and spermidine, but not spermine, were decreased in the apparently normal rectal mucosa of participants in a manner dependent on the oral DFMO dose. Spermidine:spermine ratios in rectal mucosa were also reduced in a DFMO dose-dependent manner in this study.

These studies validate measures of colorectal tissue polyamine contents as surrogate markers of DFMO effect for colon cancer chemoprevention trials. The utility of measurement of polyamine contents as marker of DFMO effect in chemoprevention trials is now being demonstrated for other tissue sites. Alberts and colleagues *(44)* found that DFMO reduced the number of premalignant actinic keratosis lesions and actinic keratosis spermidine contents in a skin cancer chemoprevention trial. We have found that DFMO treatment for 1 mo was associated with a statistically significant decrease in prostatic spermine contents of men undergoing prostate surgical procedures for several indications. The ability of DFMO to suppress tissue spermine appears

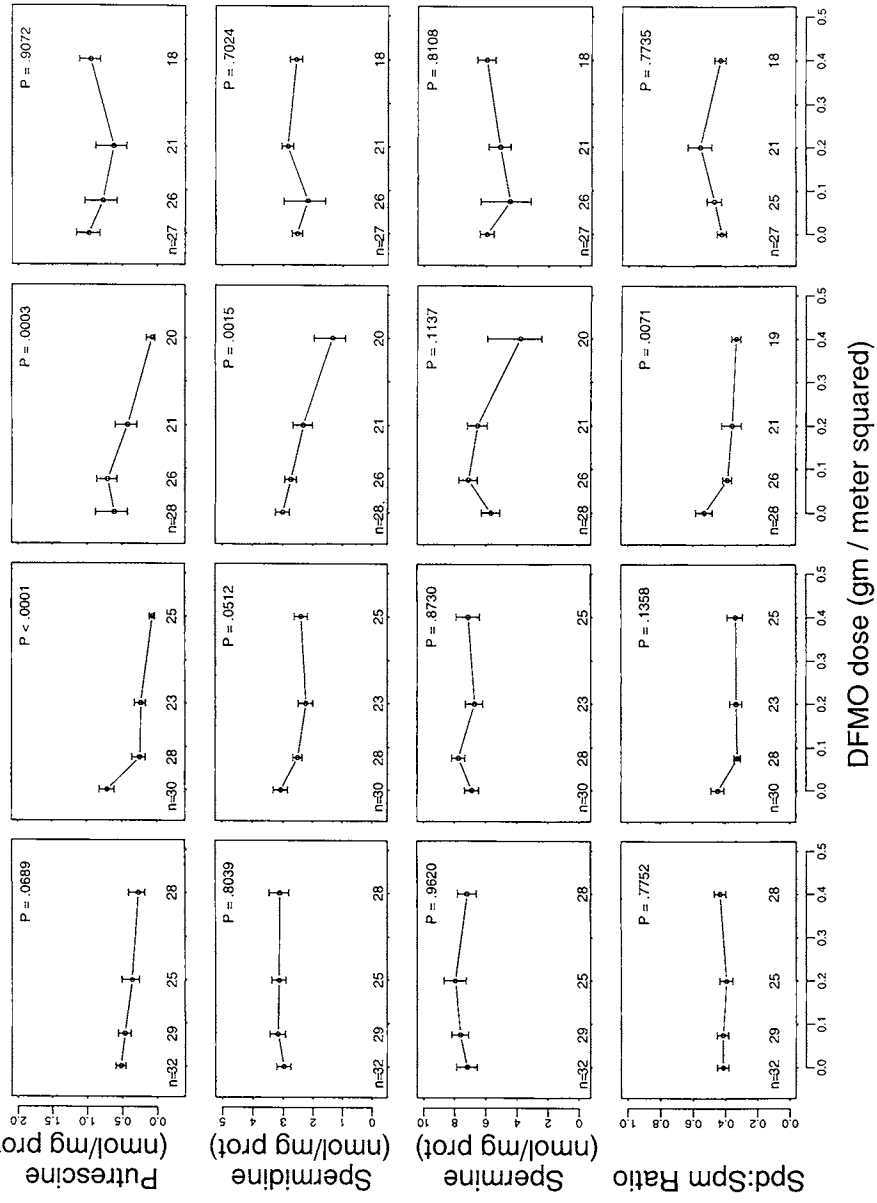

Fig. 4. Polyamine content in rectal mucosal biopsy specimens by different DFMO dosage groups (0.075, 0.20, and 0.40 g/m^2/d) over time. Values shown for putrescine, spermidine, and spermine are in mmol/mg protein; Spd:Spm = spermidine:spermine ratio; Prot = protein. *p* values are given for the test for linear trend. (Reprinted from ref. *43*.)

to be unique to the prostate, although our initial study included only a small number of men *(27)*. We have not observed DFMO-related reductions in levels of spermine in other human tissues. In a larger, randomized study soon to be reported, we have corroborated our initial finding of a reduction in prostate spermine by DFMO. The mechanism for the tissue-specific reduction of spermine by DFMO is unknown, but may be related to factors that regulate polyamine pool sizes in various tissues. Human prostate contains dramatically higher levels of spermine, relative to other polyamines, compared with other epithelial tissues. For example, levels of spermine are in the range of 3–8 nmol/mg soluble protein in apparently normal squamous esophagus, small intestine, and colon *(10,43)*. Spermidine:spermine ratios are in the range of 0.2–0.8 in these tissues. In apparently normal prostate, average spermine values exceed 20 nmol/mg soluble protein, and spermidine:spermine ratios are less than 0.05 *(27)*.

9. Genetic Variability and Polyamine Pools

A recent study of genetic variability affecting ODC expression has provided evidence that increased polyamine synthesis has a causative role in human colon cancer, rather than being a purely associative effect *(45)*. A single nucleotide polymorphism (SNP), 316 nucleotides downstream of the ODC transcriptional start site, was found to be functionally significant. This SNP is located between two consensus E-boxes in the promoter region. The transcriptional activator c-MYC and the transcriptional repressor MAD1 bind to these elements and modulate promoter activity. The frequency of this SNP has been measured in several groups of people, including participants in one colon cancer prevention trial. Fifty-five percent of participants in this trial, all of whom previously had a colon polyp, were homozygous G at this locus; 35% were heterozygous G/A, and 10% were homozygous A. The transcriptional repressor Mad1, a c-Myc antagonist, selectively repressed *ODC* transcription in an A allele-specific manner. Finally, it was found that the A allele was associated with a statistically significant reduction in risk of colon polyp recurrence. The functional significance of this and other SNPs in the *ODC* gene and their potential relationship to risk of colon and other epithelial cancers, are under active investigation by others and us. This SNP has been associated with risk of prostate cancer in a case control study *(46)*. Evaluation of polyamine contents in colon and prostate tissues in participants of our cancer chemoprevention studies, as a function of ODC genotype, is in progress. Genetic variability affecting polyamine metabolic genes could account for interindividual variations in tissue polyamine contents.

10. Polyamine Pools as Biomarkers for Agents Other Than DFMO

Measurement of tissue polyamine contents may be useful biomarkers for agents other than DFMO. Treatments that induce polyamine acetylation can deplete intracellular polyamine contents, since monoacetylspermidine and diacetylspermine are substrates for the diamine exporter (DAX) *(47)*. Polyamine analogs that induce SSAT also deplete intracellular spermidine and spermine under certain conditions *(48,49)*. The nonsteroidal anti-inflammatory drug (NSAID) sulindac, a nonselective inhibitor of both cyclo-oxygenase-1 and -2, is used to treat patients with FAP and is under active

investigation as a colon cancer chemoprevention agent in combination with DFMO *(38)*. Gene expression profiling of NSAID-treated cultured cells identified altered levels of a number of genes, including several involved in polyamine metabolism. Sulindac suppressed steady state levels of RNA encoding ODC, and increased the levels of SSAT RNA in both human cells in culture and in mice *(50)*. The mechanism of SSAT induction by sulindac involved the activation of PPARγ by the sulfone metabolite of sulindac *(16)*. This metabolite lacks the ability to inhibit cyclo-oxygenase activity, suggesting that the mechanism of PPARγ activation occurs via a cyclo-oxygenase-independent mechanism. Activated PPARγ subsequently binds to a response element near the start site of SSAT transcription. Sulindac sulfone depletes polyamine contents in human cells in culture and induces apoptosis. Cells can be rescued from sulindac sulfone-induced apoptosis by exogenous putrescine. Consistent with these results, sulindac reduced polyamine contents in intestinal mucosa of mice, especially in those treated in combination with DFMO (unpublished results, manuscript submitted). Sulindac reduced intestinal tumor number in $Apc^{Min/+}$ mice in a dose-dependent manner. The suppression of intestinal carcinogenesis by sulindac could be partially reversed by supplementing the drinking water with 1% putrescine. Mice drinking putrescine-supplemented water displayed increased intestinal putrescine concentrations. Aspirin, another NSAID, also induces SSAT promoter activity and RNA expression and causes a reduction in polyamine contents of human cells in culture *(45)*. These results indicate that several NSAIDs affect polyamine metabolic gene expression and exert at least a part of their inhibitory effects on intestinal carcinogenesis via polyamine-dependent mechanisms. Sulindac and DFMO act additively to suppress the growth of colon cancer cells in culture. In sum, these data support the rationale for combination chemoprevention of epithelial cancers using NSAIDS with DFMO. Target tissue polyamine contents may be useful predictive markers to assess the effects of combination cancer chemoprevention regimens using NSAIDS in combination with agents that affect polyamine metabolism, or in combination cancer chemotherapy trials employing certain cytotoxic drugs in combination with polyamine analogs *(51)*.

11. Buccal Mucosal Cells are not Surrogates of Other Intestinal Epithelia

Our studies have validated tissue polyamine contents as predictive markers of DFMO effect. However, obtaining tissue for assessment is often difficult, requiring substantial approvals from regulatory and other agencies. Tissue acquisition procedures often cause patient discomfort and limit the number of patients willing to provide specimens for analysis. Consequently, investigators have considered surrogate tissues that might be easier to access as alternative compartments in which to measure polyamine parameters. One of our earliest studies involved the measurement of ODC enzyme activity and polyamine contents in buccal mucosa cells *(52)*. These cells had the distinct advantage that they could be obtained in relatively large quantities by simply brushing the oral cavity with a soft toothbrush, and collecting cells in an oral wash. ODC enzyme activity and polyamine contents were easily measured using methods described in Subheading 5. We noticed, however, that ODC activities varied dramatically depending on the daily time of collection of cells. Further, putrescine

was the predominant polyamine detectable in these cells, whereas spermidine and spermine were the predominant polyamines detected in colorectal mucosal tissue of human volunteers. Buccal mucosa cells are in a cellular compartment that is rapidly turning over. We discovered that the majority of cells collected were not viable, based on assessment by vital dye exclusion methods. Several lines of evidence convinced us that the ODC enzyme activity and polyamine contents measured in our buccal mucosal preparations were predominantly of bacterial origin. Electron microscopy revealed that oral bacteria were attached to the dead buccal mucosal cells. Mouth washes with DFMO failed to influence ODC enzyme activities or polyamine contents, whereas similar washing with antiseptics reduced both ODC enzyme activities and polyamine contents. It was known that bacterial ODC is sufficiently different from mammalian ODC and is not suppressed by DFMO. The inhibition of ODC enzyme activity and polyamine contents by antiseptics in buccal mucosal, but not other human cells, was consistent with a bacterial origin of the polyamine parameters we were measuring. The marked dependence of especially ODC enzyme activity likely reflected stimulation of bacterial growth by dietary intake. Thus we concluded that buccal mucosal cells were not reliable surrogates for comparison of polyamine contents in other intestinal epithelia.

12. Urinary Polyamines as Potential Prognostic and Predictive Markers

Polyamine contents in serum and urine have also been measured as potential prognostic markers of disease or predictive markers of therapeutic interventions. Polyamine contents in serum would derive, as export products, from circulating blood cells and other body tissues. Generally, investigators fail to detect changes in serum polyamine contents that are either prognostic for disease progression or predictive of therapeutic responses. Urinary polyamine contents have been correlated with the presence of several cancers and responses of certain cancers to therapeutic interventions. Early studies inconsistently found increased urinary levels of unmodified and monoacetylated polyamines in cancer patients *(35)*. Assessment of urinary polyamine contents in patients participating in a chemoprevention trial failed to detect any effects of DFMO on either specific polyamine contents or spermidine:spermine ratios *(53)*. Most HPLC methods used in early studies of urinary polyamines required free primary amines for detection. Diacetylspermidine and diacetylspermine were undetectable by these methods. Recently, methods have been developed to detect diacetylated forms of spermidine and spermine. Notably, work by Kawakita and colleagues have found that high levels of diacetylspermine are found in the urine of cancer patients *(54–57)*. Urinary levels of diacetylspermine are a more sensitive marker, compared with carcinoembryonic antigen, of several epithelial cancers. Consideration of basic mechanisms of polyamine export supports the contention that extracellular mono- and diacetylpolyamines would be potential markers of elevated polyamine synthesis. Elevated intracellular polyamine pools activate the expression of SSAT, both transcriptionally and posttranscriptionally. SSAT will monoacetylate spermidine and will diacetylate spermine. Both monoacetyl spermidine and diacetylspermine are substrates for export by DAX in mammalian cells, as depicted in Fig. 5.

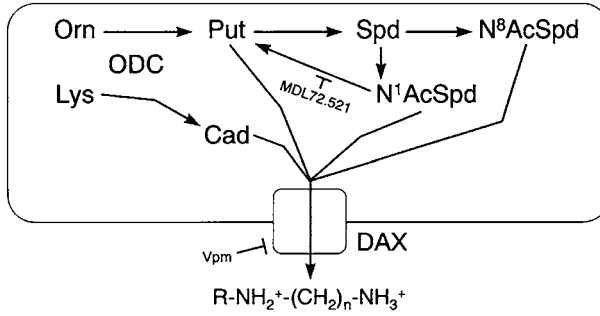

Fig. 5. Relationships between metabolism of intracellular amines and export of specific amines by the diamine exporter (DAX). Decarboxylation of lysine (Lys) and ornithine (Orn) by ODC produces the diamines cadaverine (Cad) and putrescine (Put), respectively. Putrescine is the precursor for spermidine (Spd) formation, which can be acetylated at either the N^1 (N^1AcSpd) or N^8 (N^8AcSpd) positions. MDL 72.521 is an inhibitor of the FAD-dependent polyamine oxidase (PAO), and verapamil (Vpm) is an inhibitor of DAX. This figure includes the general chemical structure of the amines exported by DAX. (Reprinted with permission from ref. *47*.)

Elevated levels of polyamine metabolic genes, including both *ODC* and *SSAT* in prostate cancers, will conceivably lead to enhanced export of diacetylspermine. Depending on its fate after export, this amine could be detected in a patient's urine. Studies of polyamine export, which used inverted plasma membrane vesicles, indicated that unmodified polyamines were poor substrates for DAX *(47)*. Thus, unmodified spermidine and spermine would find their way into urine either as a consequence of cell disruption or DAX-independent export mechanisms. Diacetylspermine appears to be the predominant form of urinary polyamines, and levels of this amine appear to increase significantly as a consequence of carcinogenesis. The studies of Kawakita and colleagues suggest that urinary levels of diacetylspermine may be a useful prognostic marker for several epithelial cancers. Whether measurement of urinary diacetylspermine can be used as a predictive marker of DFMO effect in cancer chemoprevention remains to be determined. Providing urine samples may be more widely acceptable to the general public, compared with allowing invasive procedures to obtain tissue samples. Thus, the search for surrogates for measures of tissue polyamine pools continues.

13. Summary and Conclusions

Measures of polyamine metabolism include measurements of gene products that regulate polyamine metabolism and sizes of individual polyamine pools. Several genes involved in polyamine metabolism are known targets of pathways regulating normal growth and development and tissue repair and have been implicated in carcinogenesis. However, assays that measure changes in these gene products have not proven to be reliable markers of cancer progression. This shortcoming relates, in part, to substantial variability in the values of these parameters between individuals for any specific tissue. Measures of polyamine pool size are also variable for any given tissue between individuals. However, interindividual variability in measures of polyamine pool size are less

than for other polyamine parameters. Measures of changes in polyamine pool sizes are also validated as markers of effect for the cancer chemopreventive agent DFMO. The spermidine:spermine ratio appears to be the most reliable marker for DFMO in studies of colon cancer chemoprevention. Current cancer chemoprevention trials can use measures of polyamine pools to identify the lowest, and thus probably safest, DFMO dose that will confirm biochemical activity of DFMO in a desired target tissue. Measures of polyamine pool sizes may also have relevance to selection of adequate doses of other agents, including some NSAIDS. Future studies should evaluate markers of polyamine metabolism in sources other than target tissues, as these are difficult to obtain. Urinary levels of diacetylated polyamines may prove to be useful as markers of cancer progression and as predictive markers for drugs affecting polyamine metabolism.

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References

1. Bello-Fernandez, C., Packham, G., and Cleveland, J. L. (1993) The ornithine decarboxylase gene is a transcriptional target of c-Myc. *Proc. Natl. Acad. Sci. USA* **90,** 7804–7808.
2. Nilsson, J. A., Maclean, K. H., Keller, U. B., Pendeville, H., Baudino, T. A., and Cleveland, J. L. (2004) Mnt loss triggers Myc transcription targets, proliferation, apoptosis, and transformation. *Mol. Cell Biol.* **24,** 1560–1569.
3. He, T. C., Sparks, A. B., Rago, C., et al. (1998) Identification of c-MYC as a target of the APC pathway. *Science* **281,** 1509–1512.
4. Erdman, S. H., Ignatenko, N. A., Powell, M. B., et al. (1999) APC-dependent changes in expression of genes influencing polyamine metabolism, and consequences for gastrointestinal carcinogenesis, in the Min mouse. *Carcinogenesis* **20,** 1709–1713.
5. Fultz, K. E. and Gerner, E. W. (2002) APC-dependent regulation of ornithine decarboxylase in human colon tumor cells. *Mol. Carcinogen.* **34,** 10–18.
6. Taipale, J. and Beachy, P. A. (2001) The Hedgehog and Wnt signalling pathways in cancer. *Nature* **411,** 349–354.
7. Vied, C., Halachmi, N., Salzberg, A., and Horabin, J. I. (2003) Antizyme is a target of sex-lethal in the Drosophila germline and appears to act downstream of hedgehog to regulate sex-lethal and cyclin B. *Dev. Biol.* **253,** 214–229.
8. Pendeville, H., Carpino, N., Marine, J. C., et al. (2001) The ornithine decarboxylase gene is essential for cell survival during early murine development. *Mol. Cell Biol.* **21,** 6549–6558.
9. Seshadri, T. and Campisi, J. (1990) Repression of c-fos transcription and an altered genetic program in senescent human fibroblasts. *Science* **247,** 205–209.
10. Gerner, E. W., Garewal, H. S., Emerson, S. S., and Sampliner, R. E. (1994) Gastrointestinal tissue polyamine contents of patients with Barrett's esophagus treated with alpha-difluoromethylornithine. *Cancer Epidemiol. Biomarkers Prev.* **3,** 325–330.
11. Wang, J. Y. and Johnson, L. R. (1991) Polyamines and ornithine decarboxylase during repair of duodenal mucosa after stress in rats. *Gastroenterol.* **100,** 333–343.

12. D'Orazio, D., Muller, P. Y., Heinimann, K., et al. (2002) Overexpression of Wnt target genes in adenomas of familial adenomatous polyposis patients. *Anticancer Res.* **22,** 3409–3414.
13. Giardiello, F. M., Hamilton, S. R., Hylind, L. M., Yang, V. W., Tamez, P., and Casero, R. A., Jr. (1997) Ornithine decarboxylase and polyamines in familial adenomatous polyposis. *Cancer Res.* **57,** 199–201.
14. Erdman, S. H., Wu, H. D., Hixson, L. J., Ahnen, D. J., and Gerner, E. W. (1977) Assessment of mutations in Ki-*ras* and *p53* in colon cancers from azoxymethane- and dimethylhydrazine-treated rats. *Mol. Carcinogen.* **19,** 137–144.
15. Ignatenko, N. A., Babbar, N., Mehta, D., Casero, R. A., Jr., and Gerner, E. W. (2004) Suppression of polyamine catabolism by activated Ki-ras in human colon cancer cells. *Mol. Carcinogen.* **39,** 91–102.
16. Babbar, N., Ignatenko, N. A., Casero, R. A, Jr., and Gerner, E. W. (2003) Cyclooxygenase-independent induction of apoptosis by sulindac sulfone is mediated by polyamines in colon cancer. *J. Biol. Chem.* **278,** 47,762–47,775.
17. O'Brien, T. G., Simsiman, R. C., and Boutwell, R. K. (1975) Induction of the polyamine-biosynthetic enzymes in mouse epidermis by tumor-promoting agents. *Cancer Res.* **35,** 1662–1670.
18. Weeks, C. E., Herrmann, A. L., Nelson, F. R., and Slaga, T. J. (1982) alpha-Difluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase, inhibits tumor promoter-induced polyamine accumulation and carcinogenesis in mouse skin. *Proc. Natl. Acad. Sci. USA* **79,** 6028–6032.
19. Bettuzzi, S., Davalli, P., Astancolle, S., et al. (2000) Tumor progression is accompanied by significant changes in the levels of expression of polyamine metabolism regulatory genes and clusterin (sulfated glycoprotein 2) in human prostate cancer specimens. *Cancer Res.* **60,** 28–34.
20. Ellwood-Yen, K., Graeber, T. G., Wongvipat, J., et al. (2003) Myc-driven murine prostate cancer shares molecular features with human prostate tumors. *Cancer Cell* **4,** 223–238.
21. Gupta, S., Ahmad, N., Marengo, S. R., MacLennan, G. T., Greenberg, N. M., and Mukhtar, H. (2000) Chemoprevention of prostate carcinogenesis by alpha-difluoromethylornithine in TRAMP mice. *Cancer Res.* **60,** 5125–5133.
22. Hayashi, S., Murakami, Y., and Matsufuji, S. (1996) Ornithine decarboxylase antizyme: a novel type of regulatory protein. *Trends Biochem. Sci.* **21,** 27–30.
23. Wang, Y., Devereux, W., Woster, P. M., and Casero, R. A., Jr. (2001) Cloning and characterization of the mouse polyamine-modulated factor-1 (mPMF-1) gene: An alternatively spliced homologue of the human transcription factor. *Biochem. J.* **359,** 387–392.
24. Sakata, K., Kashiwagi, K., and Igarashi, K. (2000) Properties of a polyamine transporter regulated by antizyme. *Biochem. J.* **347,** 297–303.
25. Davis, R. H., Morris, D. R., and Coffino, P. (1992) Sequestered end products and enzyme regulation: the case of ornithine decarboxylase. *Microbiol. Rev.* **56,** 280–290.
26. Hixson, L. J., Emerson, S. S., Shassetz, L. R., and Gerner, E. W. (1994) Sources of variability in estimating ornithine decarboxylase activity and polyamine contents in human colorectal mucosa. *Cancer Epidemiol. Biomarkers Prev.* **3,** 317–323.
27. Simoneau, A. R., Gerner, E. W., Phung, M., McLaren, C. E., and Meyskens, F. L., Jr. (2001) Alpha-difluoromethylornithine and polyamine levels in the human prostate: results of a phase IIa trial. *J. Natl. Cancer Inst.* **93,** 57–59.
28. Luk, G. D. and Baylin, S. B. (1984) Ornithine decarboxylase as a biologic marker in familial colonic polyposis. *N. Engl. J. Med.* **311,** 80–83.

29. Kingsnorth, A. N., Lumsden, A. B., and Wallace, H. M. (1984) Polyamines in colorectal cancer. *Br. J. Surg.* **71,** 791–794.
30. Hixson, L. J., Garewal, H. S., McGee, D. L., et al. (1993) Ornithine decarboxylase and polyamines in colorectal neoplasia and mucosa. *Cancer Epidemiol. Biomarkers Prev.* **2,** 369–374.
31. Garewal, H. S., Gerner, E. W., Sampliner, R. E., and Roe, D. (1998) Ornithine decarboxylase and polyamine levels in columnar upper gastrointestinal mucosae in patients with Barrett's esophagus. *Cancer Res.* **48,** 3288–3291.
32. Braverman, D. Z., Stankiewicz, H., Goldstein, R., Patz, J. K., Morali, G. A., and Jacobsohn, W. Z. (1990) Ornithine decarboxylase: An unreliable marker for the identification of population groups at risk for colonic neoplasia. *Am. J. Gastroenterol.* **85,** 723–726.
33. Desai, T. K., Parikh, N., Bronstein, J. C., Luk, G. D., and Bull, A.W. (1992) Failure of rectal ornithine decarboxylase to identify adenomatous polyp status. *Gastroenterol.* **103,** 1562–1567.
34. Sandler, R. S., Ulshen, M. H., Lyles, C. M., McAuliffe, C. A., and Fuller, C. R. (1992) Rectal mucosal ornithine decarboxylase activity is not a useful marker of risk for colorectal neoplasia. *Dig. Dis. Sci.* **37,** 1718–1724.
35. Wallace, H. M. and Caslake, R. (2001) Polyamines and colon cancer. *Eur. J. Gastroenterol. Hepatol.* **13,** 1033–1039.
36. Einspahr, J. G., Alberts, D. S., Gapstur, S. M., Bostick, R. M., Emerson, S. S., and Gerner, E. W. (1997) Surrogate end-point biomarkers as measures of colon cancer risk and their use in cancer chemoprevention trials. *Cancer Epidemiol. Biomarkers Prev.* **6,** 37–48.
37. Meyskens, F. L., Jr. and Gerner, E. W. (1999) Development of difluoromethylornithine (DFMO) as a chemoprevention agent. *Clin. Cancer Res.* **5,** 945–951.
38. Gerner, E. W. and Meyskens, F. L., Jr. (2004) Polyamines and cancer: Old molecules, new understanding. *Nat. Rev. Cancer* **4,** 781–792.
39. McCann, P. P., Pegg, A. E., and Sjoerdsma, A. (1987) *Inhibition of Polyamine Metabolism, Biological Significance and Basis for New Therapies.* Academic Press, Inc., Orlando, FL.
40. Gerner, E. W. and Mamont, P. S. (1986) Restoration of the polyamine contents in rat hepatoma tissue-culture cells after inhibition of polyamine biosynthesis. Relationship with cell proliferation. *Eur. J. Biochem.* **156,** 31–35.
41. Love, R. R., Carbone, P. P., Verma, A. K., et al. (1993) Randomized phase I chemoprevention dose-seeking study of alpha-difluoromethylornithine. *J. Natl. Cancer Inst.* **85,** 732–737.
42. Meyskens, F. L., Jr., Emerson, S. S., Pelot, D., et al. (1994) Dose de-escalation chemoprevention trial of alpha- difluoromethylornithine in patients with colon polyps. *J. Natl. Cancer Inst.* **86,** 1122–1130.
43. Meyskens, F. L., Jr., Gerner, E W., Emerson, S., et al. (1998) Effect of alpha-difluoromethylornithine on rectal mucosal levels of polyamines in a randomized, double-blinded trial for colon cancer prevention. *J. Natl. Cancer Inst.* **90,** 1212–1218.
44. Alberts, D. S., Dorr, R. T., Einspahr, J. G., et al. (2000) Chemoprevention of human actinic keratoses by topical 2-(difluoromethyl)-dl-ornithine. *Cancer Epidemiol. Biomarkers Prev.* **9,** 1281–1286.
45. Martinez, M. E., O'Brien, T. G., Fultz, K. E., et al. (2003) Pronounced reduction in adenoma recurrence associated with aspirin use and a polymorphism in the ornithine decarboxylase gene. *Proc. Natl. Acad. Sci. USA* **100,** 7859–7864.
46. Visvanathan, K., Helzlsouer, K. J., Boorman, D. W., et al. (2004) Association among an ornithine decarboxylase polymorphism, androgen receptor gene (CAG) repeat length and prostate cancer risk. *J. Urol.* **171,** 652–655.

47. Xie, X., Gillies, R. J., and Gerner, E. W. (1997) Characterization of a diamine exporter in Chinese hamster ovary cells and identification of specific polyamine substrates. *J. Biol. Chem.* **272,** 20,484–20,489.
48. Casero, R. A., Jr. and Pegg, A. E. (1993) Spermidine/spermine N1-acetyltransferase—the turning point in polyamine metabolism. *FASEB J.* **7,** 653–661.
49. Casero, R. A., Jr., Wang, Y., Stewart, T. M., et al. (2003) The role of polyamine catabolism in anti-tumour drug response. *Biochem. Soc. Trans.* **31,** 361–365.
50. Gerner, E. W., Ignatenko, N. A., and Besselsen, D. G. (2003) Preclinical models for chemoprevention of colon cancer. *Recent Res. Cancer Res.* **163,** 58–71; discussion 264–266.
51. Choi, W., Gerner, E. W., Ramdas, L., et al. (2005) Combination of 5-fluorouracil and diethynorspermine markedly activates SSAT expression, depletes polyamines and synergistically induces apoptosis in colon cancer cells. *J. Biol. Chem.* **280,** 3295-3304.
52. Boyle, J. O., Meyskens, F. L., Jr., Garewal, H. S., and Gerner, E. W. (1992) Polyamine contents in rectal and buccal mucosae in humans treated with oral difluoromethylornithine. *Cancer Epidemiol. Biomarkers Prev.* **1,** 131–135.
53. Fabian, C. J., Kimler, B. F., Brady, D. A,. et al. (2002) A phase II breast cancer chemoprevention trial of oral alpha-difluoromethylornithine: breast tissue, imaging, and serum and urine biomarkers. *Clin. Cancer Res.* **8,** 3105–3117.
54. Hiramatsu, K., Sugimoto, M., Kamei, S., et al. (1997) Diagnostic and prognostic usefulness of N1,N8-diacetylspermidine and N1,N12-diacetylspermine in urine as novel markers of malignancy. *J. Cancer Res. Clin. Oncol.* **123,** 539–545.
55. Hiramatsu, K., Miura, H., Kamei, S., Iwasaki, K., and Kawakita, M. (1998) Development of a sensitive and accurate enzyme-linked immunosorbent assay (ELISA) system that can replace HPLC analysis for the determination of N1,N12-diacetylspermine in human urine. *J. Biochem.* **124,** 231–236.
56. Kobayashi, M., Samejima, K., Hiramatsu, K., and Kawakita, M. (2002) Mass spectrometric separation and determination of N1,N12-diacetylspermine in the urine of cancer patients. *Biol. Pharm. Bull.* **25,** 372–374.
57. Kawakita, M., Hiramatsu, K., Sugimoto, M., Takahashi, K., and Toi, M. (2004) [Clinical usefulness of urinary diacetylpolyamines as novel tumor markers]. *Rinsho Byori Jpn. J. Clin. Pathol.* **52,** 321–327.

III

Polyamines in Cell Motility and Cell–Cell Interactions

20

Polyamines and Cytoskeletal Regulation During Intestinal Epithelial Restitution

Ramesh M. Ray and Leonard R. Johnson

1. Introduction

Damage to gastrointestinal epithelia can result from infection (ulcer), chemical agents (alcohol, drugs), or mechanical forces (stretching), and immediate repair is required to restore the epithelial barrier against luminal antigens. The mucosa of the gastrointestinal tract has the unique ability to repair itself rapidly after damage. Mucosal repair consists of two phases. Early mucosal restitution is the rapid re-establishment of epithelial integrity and continuity after superficial injury before cell proliferation or an extensive inflammatory response occurs *(1)*. It comprises sloughing of the damaged cells and migration of remaining viable cells over the denuded lamina propria *(2,3)*. The second phase involves replacement of the lost cells by mitosis and does not begin until 24 h or so after injury *(1)*. The process of early mucosal restitution was originally described for the stomach, but later Fiel et al. *(4)* and Moore et al. *(5)* have shown a similar process for the small intestine.

The mucosal epithelium of the alimentary tract represents a crucial barrier to a broad spectrum of noxious and immunogenic substances within the intestinal lumen. Impairment of the integrity of the mucosal epithelial barrier is observed in the course of various intestinal disorders, including inflammatory bowel diseases, celiac disease, intestinal infections, and various other diseases. Furthermore, even under physiological conditions proteases, residential flora, dietary compounds, or other factors may cause temporary damage to the epithelial surface. Healing of the intestinal epithelium is regulated by a complex network of highly divergent factors, among them a broad spectrum of structurally distinct regulatory peptides that have been identified within the mucosa. These regulatory peptides, conventionally designated as growth factors and cytokines, play an essential role in regulating differential epithelial cell functions to preserve normal homeostasis and integrity of the intestinal mucosa. In addition, a number of other peptides, such as those in the extracellular matrix and blood clotting factors, as well as nonpeptides including phospholipids, short chain fatty acids, adenine

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nucleotides, trace elements, and pharmacological agents, modulate intestinal epithelial repair. Some of these molecules may be released by platelets, adjacent stromal cells, inflammatory cells, or injured epithelial and nonepithelial cells. Enhancement of repair mechanisms by regulatory factors may provide future approaches for the treatment of diseases that are characterized by damage to the epithelial surface.

Polyamines have been shown to be essential for various processes, including cell proliferation, migration, and apoptosis both in animal models and in in vitro cell culture models *(6–8)*. The role of polyamines in the regulation of cell proliferation and apoptosis has been discussed in detail elsewhere in this book. Because migration is involved in restitution, it is important to understand the mechanism by which polyamines affect this process. Using stress and hypertonic NaCl models for mucosal injury in rats, it has been shown that polyamines are essential for the healing of gastric and intestinal lesions *(8–10)*. The polyamines spermidine, spermine, and their precursor putrescine, are found in virtually all cells of higher eukaryotes and are intimately involved in, and required for, cell growth and proliferation *(11,12)*. Intracellular polyamine levels are highly regulated and depend on the activity of ornithine decarboxylase (ODC), which catalyzes the first rate-limiting step in polyamine biosynthesis *(12,13)*. An increase in ODC activity is one of the earliest events associated with the induction of cellular proliferation and depletion of polyamines by DL-α-difluoromethylornithine (DFMO), a specific inhibitor and irreversible inhibitor of ODC, attenuates trophic responses in tissues and cultured cells *(14–17)*.

ODC activity in the mucosa of the small intestine is increased after partial resection *(18)*, during lactation *(19)* during the third week of life at the time of weaning in rats *(20)*, and after obstruction of the lumen *(21)*—all instances associated with mucosal growth. DFMO prevents the accumulation of polyamines and growth of the mucosa in each of these models *(18–21)*. In both gastric *(8,22)* and duodenal *(22,23)* mucosa, ODC activity increases significantly in response to the production of stress ulcers. In the same models, the mucosal polyamine content also increases, and DFMO prevents the increases in ODC and polyamines *(8,22,23)*. Although polyamine depletion did not increase the severity of the lesions, it prevented, almost entirely, the 80–90% healing that occurred within the 24-h period after damage *(8,23–25)*. Oral administration of any of the polyamines immediately after the period of stress increased the rate of normal healing and prevented the inhibition of repair caused by DFMO in both gastric *(24)* and duodenal *(25)* mucosa. Because polyamines are required for cell division, some of their beneficial effects were a result of this process; however, substantial and significant healing occurred in polyamine-depleted rats given exogenous spermidine and spermine within 12 h after damage *(24,25)*. Because regeneration by proliferation requires at least 24 h *(1,26,27)*, it was obvious that the polyamines are essential to the process of early mucosal restitution and cell migration, as well as proliferation.

2. Mechanism of Cell Migration

Cell migration involves a coordinated cycle of plasma membrane protrusion at the leading edge, adhesion formation to stabilize the protrusion, destabilization of older adhesion sites at the rear of the cell, and stress fiber contraction against adhesions for

cell body movement *(28,29)*. The initial response of a cell to a migration-promoting stimulus is to polarize and extend protrusions in the direction of migration. These protrusions, which can be broad and large or spikelike structures, called lamellipodia and filopodia, respectively, are thought to result from actin filament (F-actin) polymerization at the plasma membrane. In lamellipodia, actin filaments form a branching network, whereas they form parallel bundles in the filopodia. These protrusive structures are stabilized by adhering to the extracellular matrix (ECM) or to adjacent cells via transmembrane proteins linked to the actin cytoskeleton. Adhesion to the ECM provides traction sites for the forward movement of the cell. Thus dynamic assembly and disassembly of these adhesions plays a crucial role in determining the direction and rate of cell motility. Using fluorescence speckle microscopy, Ponti et al. *(30)* showed that assembly and disassembly of the lamellipodial network occurred within 1–3 μm of the leading edge. It was weakly coupled to the rest of the cytoskeletal structure and promoted random protrusions. However, productive cell movement resulted from a second network colocalized with actomyosin at focal adhesions in migrating epithelial cells *(30)*.

Actin filaments consist of fast-growing "barbed ends" and slow-growing "pointed ends" imparting polarity, which is used to drive membrane protrusions. The Arp2/3 complex mediates actin polymerization in lamellipodia. Several actin-binding proteins also regulate the rate and organization of actin polymerization by modulating the pool of available monomers (G-actin) and free ends. Profilin prevents self-nucleation by binding to actin monomers and also is involved in targeting monomers to barbed ends. Filament elongation is prevented by capping proteins, resulting in decreased polymerization of new filaments in the vicinity of the plasma membrane. The ADF/cofilin group of proteins promotes disassembly of older filaments, which is required to generate actin monomers for polymerization at the leading edge. These proteins sever filaments and promote actin dissociation from the pointed end and are referred to as actin severing proteins *(31,32)*.

3. Polyamines and Migration in Intestinal Epithelial Cells

The mechanism by which polyamines influence cell migration is not clear. Polyamines affect various types of cell migration, such as human sperm motility *(33)*, metastasis of breast cancer cells *(34)*, and cell attachment to fibronectin *(35)* in addition to epithelial cell motility. Our laboratory elucidated the role of polyamines in gastrointestinal healing using an in vivo rat model. McCormack et al. *(36)* established an in vivo cell culture model of restitution using the IEC-6 cell line, a well-characterized intestinal crypt cell line derived from the rat by Quaroni et al. *(37)*. To determine the rate of migration, we used a modified model in which IEC-6 cells are plated on 35-mm plates and allowed to grow to confluence. Plates are marked at the bottom along the diameter and wounded twice with a fine, sterile, plastic microtip perpendicular to the marking. Plates are washed to remove damaged cells, replenished with fresh medium, and photographed at 0 h and at various times allowed for migration, usually 7–8 h (Fig. 1). The width of the wound covered by migrating cells is determined by National Institutes of Health imaging software and used for calculating the rate of migration *(38)*. To study the effects of polyamines on migration, cells are grown for 4 d in control medium containing

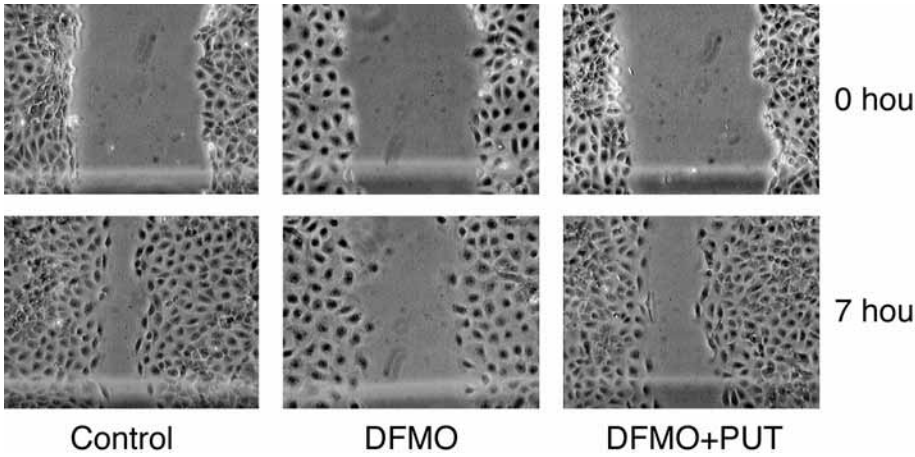

Fig. 1. Wound healing model and effect of polyamine depletion. Cell migration wound assay of IEC-6 cells grown in control, α-difluoro-ornithine (DFMO), and DFMO plus putrescine containing media as described in ref. *41* showing size of wound at 0 and 7 h after wounding. DFMO-treated cells show decreased wound closer.

5 m*M* DFMO and medium containing DFMO plus putrescine. In the presence of DFMO, intracellular putrescine disappears completely within 6 h, spermidine within 48 h, and spermine decreases to 30 to 40% by d 4 *(39)*. Exogenous addition of putrescine to DFMO containing medium serves as a control to ascertain that the effects of DFMO are owing to depletion of polyamines and not to DFMO itself. In both animal and cell culture models, polyamine depletion decreased migration by 60–70%, and addition of putrescine to DFMO-containing medium restored migration to control levels (Fig. 1). Furthermore, inhibition of *S*-adenosylmethionine decarboxylase decreased intracellular spermidine and spermine, increased putrescine, and inhibited migration, suggesting that putrescine is not required for the migration of these cells *(40)*. Examination of the actin cytoskeletal structure revealed a significant alteration in the organization of F-actin in response to polyamine depletion. In polyamine-depleted cells, F-actin localized to a heavy actin cortex with short stress fibers (Fig. 2A). Unlike cells grown under control conditions, cells grown in DFMO showed significantly less F-actin reorganization at the migrating edge (Fig. 2B). In control cells, long stress fibers of F-actin traversed the cell and lamellipodia, a characteristic feature of actively migrating cells, were evident. Total amounts of G-actin and F-actin were unchanged in control cells and those depleted of polyamines, suggesting that the remodeling of the actin cytoskeleton, rather than the ratios of the actins, was altered by polyamines *(41)*.

Attachment to the ECM is the first step in migration. To migrate, cells must form and break attachments with the ECM. The ECM binds to cell surface receptors, the integrins, resulting in their aggregation and the formation of a signaling complex, which provides attachment sites for stress fibers *(42)*. The reorganization of actin filaments into stress fibers causes a positive feedback, resulting in additional integrin clustering *(43)*. The

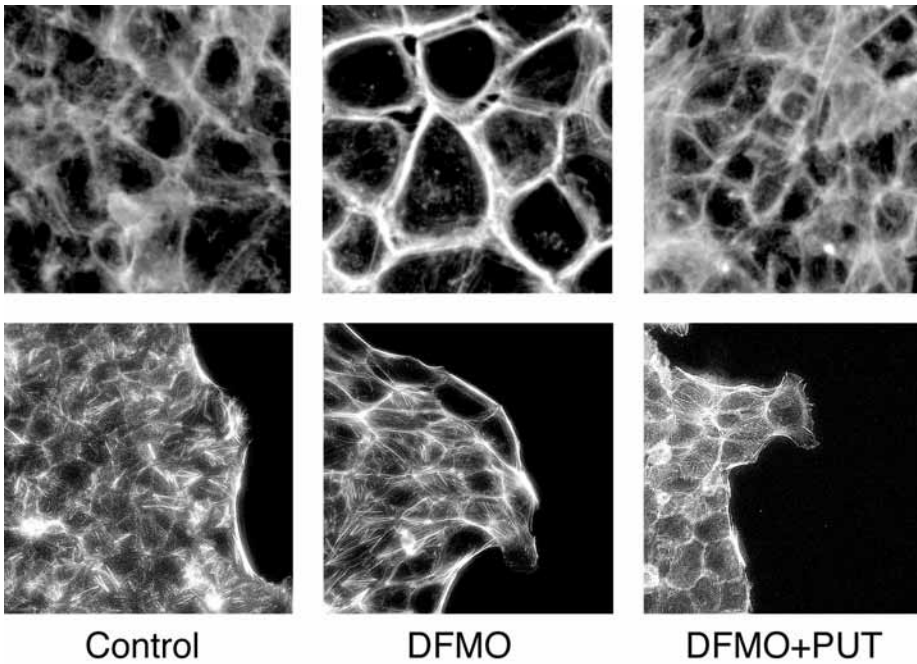

Fig. 2. Effect of polyamine depletion on the F-actin cytoskeleton. IEC-6 cells grown in control, α-difluoro-ornithine (DFMO), and DFMO plus putrescine containing media were fixed after 7 h migration, stained with rhodamine-conjugated phalloidin and observed under ultraviolet fluorescence with an inverted microscope. Control and DFMO plus putrescine groups show stress fibers in the confluent area **(A)**, and at the migrating edge **(B)**. The DFMO group has a thick cortical localization of F-actin and fewer stress fibers.

resulting transmembrane complexes of extracellular matrix proteins, integrins, cytoskeletal proteins, focal adhesion kinase, and actin are called focal adhesion complexes. Both attachment and stress fiber formation depend on focal adhesion complexes and integrin signaling. Polyamine depletion of cells significantly decreased attachment to fibronectin *(44)*, decreased clustering of integrin α1 with β2, and decreased tyrosine phosphorylation of focal adhesion kinase (FAK), specifically, the phosphorylation of tyrosine 925, the paxillin binding site *(44)*. In control cells, FAK phosphorylation occurred rapidly after attachment to the ECM, whereas it was significantly delayed in attached cells depleted of polyamines. Autophosphorylation of FAK was also significantly inhibited, as was the phosphorylation of paxillin in polyamine-depleted cells. Polyamine-depleted cells failed to spread normally after attachment, and immunocytochemistry showed little colocalization of FAK and actin compared with controls. Focal adhesion complex formation was greatly reduced in the absence of polyamines, suggesting that decreased integrin signaling may, in part, account for the decreased rates of attachment, spreading, and migration that are observed in polyamine-depleted cells.

Soluble factor and integrin signaling is relayed to the cytoskeleton by signal transduction pathways involving a subgroup of the Ras superfamily of small guanosine 5′-triphosphate-binding proteins *(45)*. The Rho (Ras homology) guanosine 5′-triphosphatases (GTPases) consist of three major types of small (21 kDa) proteins that bind GTP. Their intrinsic GTPase activity is controlled by guanine nucleotide exchange factors and GTPase-activating proteins. Rho guanine nucleotide dissociation inhibitor is an inhibitory guanine nucleotide exchange factors that prevents the dissociation of guanosine diphosphate from Rho as well as from Rac and Cdc42, the two other members of this family *(46)*. Rho and Rac regulate the polymerization of actin and subsequent formation of stress fibers and lamellipodia, respectively *(47–49)*. Cdc42 has been shown to be responsible for the formation of filopodia *(50)*.

Polyamine-deficient Chinese hamster ovary cells lack actin filaments and microtubules unless polyamines are supplied exogenously *(51)*. Inhibitors of polyamine synthesis prevent concanavalin A-induced expression of α-tubulin and β-actin messenger RNAs in mouse splenocytes *(52)*. In vitro, polyamines stimulate the rapid polymerization of G-actin and formation of F-actin, indicating a possible direct effect of polyamines on cytoskeletal organization *(53)*. Because the early phase of mucosal healing, which is the result of cell migration, requires polyamines *(23,24)* and polyamine depletion inhibits migration in the IEC-6 cell model (Fig. 1) and leads to numerous alterations in the actin cytoskeleton (Fig. 2) *(42)*, we examined the effect of polyamine depletion on the Rho proteins. Santos et al. *(54)* showed that Rho was required for migration in response to wounding or in response to growth factor stimulation. Migration was inhibited after microinjection of the Rho guanine nucleotide dissociation inhibitor, *Clostridium botulinum C3* adenosine 5′-diphosphate-ribosyltransferase toxin, or a dominant–negative form of RhoA (Rho T19N). The actin cytoskeleton was also altered in these cells in ways that were identical to those produced by polyamine depletion. Polyamine depletion caused significant reduction in RhoA protein in the cytoplasm and membrane fractions of IEC-6 cells. An approx 50% reduction in the rate of synthesis of RhoA protein was observed in polyamine-depleted cells without a reduction in the half-life of RhoA or the levels of RhoA messenger RNA *(55)*. Transfection of IEC-6 cells with constitutively active RhoA (HA-V14-RhoA) increased the rate of migration compared with cells transfected with empty vector, and dominant–negative RhoA cells exhibited almost no motility. Surprisingly, polyamine-depleted cells expressing constitutively active RhoA also had reduced rates of migration *(55)*. Polyamine depletion inhibited RhoA activity in cells transfected with vector as it did in untransfected cells. In cells expressing dominant–negative RhoA, the cortical localization of F-actin was almost identical to that observed in polyamine-depleted cells. Constitutively active RhoA was unable to restore stress fiber formation in polyamine-depleted cells. Thus, although RhoA activity is essential for the migration of intestinal epithelial cells (IECs), it is not sufficient to reverse the effects of polyamine depletion.

There is considerable signaling cross talk between Rho, Rac, and Cdc42, and each of these molecules regulates a different aspect of the motility machinery of the cell. Transfection of IEC-6 cells with constitutively active Rac1 (V12-Rac1) completely reversed the effects of polyamine depletion on migration, restored the actin cytoskeletal

structure, and returned the levels of RhoA protein to normal *(38)*. This finding also implies that polyamines do not exert their effects by interacting directly with cytoskeletal proteins. IECs expressing constitutively active Rac1 had increased basal levels of active RhoA and Cdc42, indicating that Rac1 activates RhoA and Cdc42. Constitutively active RhoA cells had increased Cdc42 activity but Rac1 activities were unchanged. Activation of Cdc42 also increased the basal activity of Rac1 and RhoA. These observations clearly indicate that Rac1 is upstream of RhoA, and that RhoA may be downstream of Cdc42. Thus, Rac1 activation is not only essential but also sufficient for cell migration. This observation provides important insights into the involvement of polyamines in migration. Polyamines either activate Rac1 directly or, more likely, are required for an upstream event for the activation of Rac1 and not for the maintenance of levels of Rac1 protein. Rac1 protein levels were unaltered in response to polyamine depletion.

Epidermal growth factor (EGF) plays a critical role in the protection and repair of the gastrointestinal mucosa *(56–58)*. EGF is produced by salivary glands *(59)* and by the gastric mucosa *(60)*, and EGF receptors are present on the epithelial cells of the gastrointestinal tract *(61,62)*. Injury-induced increases in the production of EGF and overexpression of EGF receptor (EGFR) have been observed during mucosal repair *(63,64)*. We have shown that wounding transiently increased mitogen-activated protein kinase (MAPK) activity, and that EGF further increased migration in control and polyamine-depleted cells *(65)*. However, in polyamine-depleted cells, EGF stimulated migration only to the levels observed in untreated control cells. U-0126, a strong inhibitor of mitogen-activated protein kinase kinase (MEK) 1, decreased basal as well as EGF-induced extracellular signal-regulated kinase (ERK) phosphorylation and inhibited migration, indicating that MAPK activity is required for migration. Xie et al. *(66)* reported that the EGF-mediated disassembly of focal adhesions depended on ERK activation. Klemke et al. *(67)* have shown that activated ERK can associate with, and phosphorylate, myosin light chain kinase, thereby increasing its activity and enhancing migration. Sustained ERK2 activation mediates scattering in SK-N-MC cells in response to Ret (rearranged during transfection) proto-oncogene and fibroblast growth factor *(68)*, and stimulates colony dispersion in SCC-12F cells *(69)*. In fibroblasts, U-0126 inhibits EGF-induced migration *(70)*. These reports suggest that ERK1/2 inhibition alone is sufficient to prevent migration. We have also observed that on EGF treatment, EGFR downregulation occurs within 10–15 min (unpublished observations). Therefore, we predicted that downregulation of EGFR and the transient nature of ERK activation might be rate-limiting factors in the migration of polyamine-depleted cells. We used stable IEC-6 cell lines expressing HA-tagged constitutively active (CA) and dominant–negative (DN) MEK1 to examine the effects of sustained activation and inhibition of ERK1/2 on migration. Characteristic of actively migrating cells, those expressing CA-MEK exhibited significant spreading and stress fiber formation, which were less prominent in cells transfected with empty vector *(65)*. In contrast, cells expressing DN-MEK were characterized by a significant loss of actin stress fibers essential for maintenance of cell shape, size, and migration. CA-MEK restored migration of polyamine-depleted cells to a level comparable to that of cells transfected with CA-MEK and grown in control medium. Migration of these cells was significantly higher

compared with that observed in cells transfected with empty vector. Unlike cells transfected with empty vector, those transfected with CA-MEK displayed extensive actin cytoskeletal reorganization at the migrating edge in response to polyamine depletion.

The intracellular localization of Rho GTPases is crucial for their activation. RhoA and Rac1 proteins were localized throughout the cytoplasm in cells transfected with empty vector grown in control and DFMO plus putrescine media, and aggregates of RhoA were clearly distinguishable at the cell periphery. Interestingly, in polyamine-depleted cells, the subcellular localization of RhoA and Rac1 was significantly altered. Significant amounts of RhoA protein were found in the nucleus and in the perinuclear region with decreased concentrations at the cell periphery. In contrast, in cells transfected with CA-MEK, aggregates of RhoA protein were distributed throughout the cytoplasm with relatively higher amounts at cell periphery. Surprisingly, a significant fraction of Rac1 in polyamine-depleted cells transfected with empty vector was localized in the nucleus, unlike control and DFMO plus putrescine groups in which it localized throughout the cytoplasm. Expression of CA-MEK in polyamine-depleted cells prevented the nuclear accumulation of Rac1 and increased its distribution in cytoplasm. Unlike polyamine-depleted cells transfected with empty vector, Rac1 was localized in lamellipodia of DFMO-treated cells transfected with CA-MEK. We have shown that constitutively active RhoA increased cell proliferation by preventing the transcription of p21waf/kip in IECs *(71)* and that constitutive activation of Rac1 also increased proliferation (unpublished data), suggesting additional roles for these proteins, which might explain their localization in the nucleus.

Because constitutive activation of Rac1 and MEK proved to be necessary and sufficient for the migration of polyamine-depleted cells, we examined whether MEK activated Rac1 in polyamine-depleted cells. CA-MEK expression increased Rac1 activity in control and polyamine-depleted cells, unlike cells transfected with vector, which showed decreased Rac1 activity. These results clearly demonstrate that MAPK activation is necessary for actin cytoskeletal organization, which is essential for migration.

Nonmuscle myosin II has been shown to play an important role in the regulation of the dynamics of actin cytoskeletal structure and cell shape. These changes are associated with changes in intracellular Ca^{2+} levels and its distribution in organelles *(72)*. Polyamine depletion significantly decreased levels of myosin II. Myosin II localized in the form of small patches throughout the cytoplasm instead of in its functional association with stress fibers *(73)*. Decreased intracellular Ca^{2+} resulting from downregulation of voltage gated K^+ (Kv1.1) channel expression has been shown to influence RhoA protein levels and migration in polyamine-depleted cells *(74,75)*. Levels and distribution of myosin II and levels of intracellular Ca^{2+} and RhoA protein were restored to normal by exogenous addition of putrescine to cells grown in the presence of DFMO. Furthermore ionomycin, a Ca^{2+} ionophore, also increased RhoA protein and migration in polyamine-depleted cells. However, the enzymatic activity of RhoA, which is essential to migration, was not determined in this study. It is difficult to understand how intracellular K^+ and Ca^{2+} homeostasis can be regulated by a single mechanism, which determines all or no effects on migration. Increased K^+ channel activity has been shown to increase the migration of fibroblasts, however, inhibition of K^+ channel activity also

accelerates intestinal wound healing in the human colon carcinoma cell lines, T84 and Caco-2, via both Ca^{2+}-dependent and constitutively active channels *(76)*. Pharmacological activation of K^+ channels had no effect on the repair process in those cell lines *(76)*. Furthermore, spermine and spermidine have been shown to inhibit inward rectifying K (Kir) channel activity *(77)*. Thus, in the absence of polyamines (polyamine-depleted cells), Kir channels should have higher activity because of the lack of gating by spermidine and spermine.

Polyamine depletion decreases total RhoA protein, and significantly decreases the activities of RhoA, Rac1, and Cdc42 *(54)*. Furthermore, expression of constitutively active RhoA does not restore migration in polyamine-depleted cells, indicating that normal RhoA protein levels or activity are not sufficient for migration. Therefore, increases in RhoA protein in polyamine-depleted cells in response to increased intracellular Ca^{2+} may not account for the restoration of migration. Ca^{2+} may actually regulate a step upstream from Rho that is sufficient for migration. Intracellular Ca^{2+} homeostasis and the desired levels of cytoplasmic-free Ca^{2+} depend on binding proteins known as calmodulins and storage in the endoplasmic reticulum. Sustained increases in free cytoplasmic Ca^{2+} have been shown to decrease migration and may induce apoptosis *(78–80)*. Thapsigargin, an endoplasmic reticulum Ca^{2+} ATPase inhibitor, increases Ca^{2+} in the cytoplasm by activating store operated Ca^{++} entry. Thapsigargin increased intracellular Ca^{2+} in both control and polyamine-depleted cells, but did not restore migration of polyamine-depleted cells (unpublished data). Because polyamine depletion decreases the cell number in confluent cultures of IEC-6 cells by about 40%, fewer cells occupy a unit area. Thus less Ca^{2+} might be measured in monolayers of polyamine-depleted cells without translating in to decreased Ca^{2+} levels per cell. Because intracellular Ca^{2+} has not been determined in normal and polyamine-depleted cells, its role in RhoA activation in DFMO treated cells is unclear.

4. Conclusions

The scheme in Fig. 3 depicts the signaling events reported to be associated with polyamine depletion in IECs. Inhibition of migration of these cells is associated with decreased integrin ($\alpha 2/\beta 1$) heterodimerization *(46)*, myosin distribution *(76,78)*, myosin light chain phosphorylation *(78)*, intracellular Ca^{2+} levels *(78)*, expression and activity of Kv channels *(77)*, cell attachment and spreading *(46)*, phosphorylation of FAK and paxillin *(46)*, RhoA protein levels *(54)*, and activities of RhoA, Rac1, and Cdc42 *(41,54)*. Constitutive activation of MEK1 or Rac1 *(67)* completely restores migration and RhoA protein. This suggests that MEK1 or an upstream activator is a sensor of intracellular polyamine levels. Intracellular Ca^{2+} may activate MEK1, which in turn may activate Rac1 leading to activation of RhoA and Cdc42 and restore migration in polyamine-depleted cells. Because wounding initiates migration, it is reasonable to believe that polyamines modulate signaling at a membrane receptor where most of the key molecules described are found to localize at some time and point during migration. However, it is not clear how polyamines, in response to wounding, integrate outside signals to the intracellular events leading to migration. Current evidence suggests that, after wounding, polyamines influence the intracellular events associated

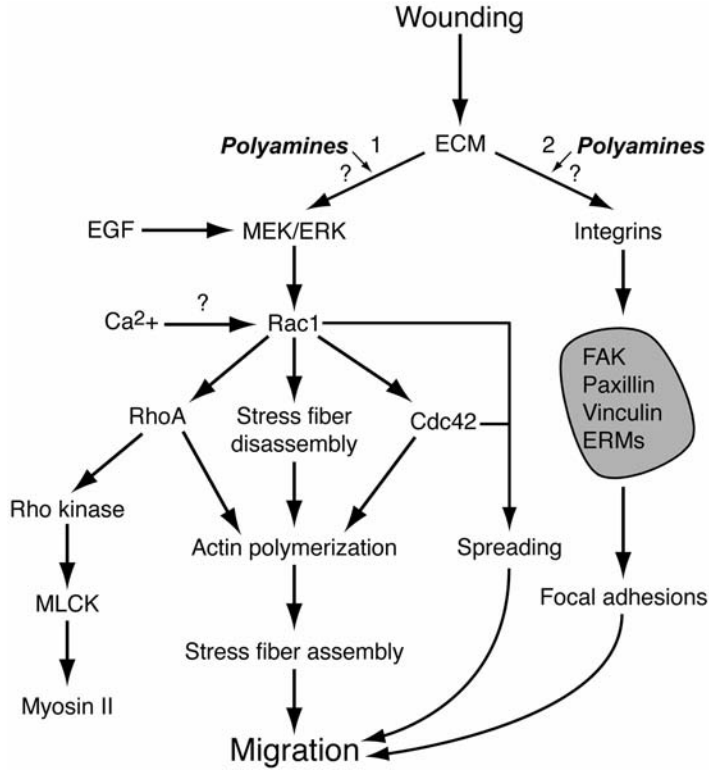

Fig. 3. Wound-induced signaling during cell migration. Steps depicted in the signaling cascade are directly or indirectly altered in response to polyamine depletion. FAK, focal adhesion kinase; ERMs, ezrin, radixin, moesin actin-binding proteins of focal adhesion complexes; MLCK, myosin light chain kinase; ECM, extracellular matrix.

with migration in two ways. First, they are involved in the activation of MEK and ERKs, which in turn activate Rac1. Second, they play a role in the organization of the focal adhesion complex via integrin ligation.

References

1. Silen, W. and Ito, S. (1985) Mechanism for rapid epithelialization of the gastric mucosal surface. *Ann. Rev. Physiol.* **47,** 217–229.
2. Ito, S., Lacy, E. R., Rutten, M. J., Critchow, J., and Silen, W. (1984) Rapid repair of injured gastric mucosa. *Scand. J. Gastroenterol.* **19,** 87–95.
3. Rutten, M. J. and Ito, S. (1983) Morphology and electrophysiology of guinea pig gastric mucosal repair in vitro. *Am. J. Physiol.* **244,** G171–G182.
4. Feil, W., Wenzi, E., Vattay, P., Starlinger, M., Sogukoglu, T., and Schiessel, R. (1987) Rapid repair of rabbit duodenal mucosa after acid injury in vivo and in vitro. *Gastroenterol.* **92,** 1973–1986.

5. Moore, R., Carlson, S., and Madara, J. L. (1989) Rapid barrier restitution in an in vitro model of intestinal epithelial injury. *Lab. Invest.* **60,** 277–284.
6. Ray, R. M., Zimmerman, B. J., McCormack, S. A., Patel, T. B., and Johnson, L. R. (1999) Polyamine depletion arrests cell cycle and induces inhibitors p21(Waf1/Cip1), p27(Kip1), and p53 in IEC-6 cells. *Am. J. Physiol. Cell Physiol.* **276,** C684–C691.
7. Ray, R. M., Viar, M. J., Yuan, Q., and Johnson, L. R. (2000) Polyamine depletion delays apoptosis of rat intestinal epithelial cells. *Am. J. Physiol. Cell Physiol.* **278,** C480–C489.
8. Wang, J.-Y. and Johnson, L. R. (1990) Role of ornithine decarboxylase in repair of gastric mucosal stress ulcers. *Am. J. Physiol.* **258,** G78–G85.
9. Wang, J.-Y. and Johnson, L. R. (1990) Gastric and duodenal mucosal ornithine decarboxylase and damage after corticosterone. *Am. J. Physiol.* **258,** G942–G950.
10. Wang, J.-Y., Viar, M. J., and Johnson, L. R. (1993) Transglutaminase in response to hypertonic NaCl-induced gastric mucosal injury in rats. *Gastroenterology* **104,** 65–74.
11. Pegg, A. E. and McCann, P. P. (1982) Polyamine metabolism and function. *Am. J. Physiol.* **243,** C212–C221.
12. Pegg, A. E. (1986) Recent advances in the biochemistry of polyamines in eukaryotes. *Biochem. J.* **243,** 249–262.
13. Tabor, C. W. and Tabor, H. (1984) Polyamines. *Ann. Rev. Biochem.* **53,** 749–790.
14. Russell, D. H. and Durie, B. G. M. (1978) Ornithine decarboxylase-a key enzyme in growth. *Proc. Cong. Res. Ther.* **8,** 43–58.
15. Russell, D. H. (1985) Ornithine decarboxylase: A key regulatory enzyme in normal and neoplastic growth. *Drug Metab. Rev.* **16,** 1–88.
16. Pegg, A. E., McGovern, K. A., and Weist, L. (1987) Decarboxylation of α-difluoromethylornithine by ornithine decarboxylase. *Biochem. J.* **241,** 305–307.
17. Seidel, E. R., Haddox, M. R., and Johnson, L. R. (1984) Polyamines in the response to intestinal obstruction. *Am. J. Physiol.* **246,** G649–G653.
18. Luk, G. D. and Baylin, S. B. (1983) Polyamines and intestinal growth-increased polyamine biosynthesis after jejunectomy. *Am. J. Physiol.* **245,** G556–G660.
19. Yang, P., Baylin, S. B., and Luk, G. D. (1984) Polyamines and intestinal growth: absolute requirement for ODC activity in adaptation during lactation. *Am. J. Physiol.* **247,** G553–G557.
20. Luk, G. D., Baylin, S. B., and Marton, L. J. (1980) Ornithine decarboxylase is important in intestinal mucosal maturation and recovery from injury in rats. *Science* **210,** 195–198.
21. Seidel, E. R., Haddox, M. R., and Johnson, L. R. (1984) Polyamines in the response to intestinal obstruction. *Am. J. Physiol.* **246,** G649–G653.
22. Wang, J.-Y. and Johnson, L. R. (1989) Induction of gastric and duodenal mucosal ornithine decarboxylase during stress. *Am. J. Physiol.* **257,** G259–G265.
23. Wang, J.-Y. and Johnson, L. R. (1991) Polyamines and ornithine decarboxylase during repair of duodenal mucosa after stress in rats. *Gastroenterol.* **100,** 333–343.
24. Wang, J.-Y. and Johnson, L. R. (1990) Luminal polyamines stimulate repair of gastric mucosal stress ulcers. *Am. J. Physiol.* **259,** G584–G592.
25. Wang, J.-Y. and Johnson, L. R. (1992) Luminal polyamines substitute for tissue polyamines in duodenal mucosal repair after stress in rats. *Gastroenterol.* **102,** 1109–1117.
26. Lipkin, M., Sherlock, P., and Bell, B. (1963) Cell proliferation kinetics in the gastrointestinal tract of man. II. Cell renewal in stomach, ileum, colon, and rectum. *Gastroenterol.* **45,** 721–729.
27. Stephens, C. R. and Leblond, C. P. (1953) Renewal of the mucous cells in the gastric mucosa of the rat. *Anat. Rec.* **115,** 231–243.

28. Lauffenburger, D. A. and Horwitz, A. F. (1996) Cell migration: A physically integrated molecular process. *Cell* **84,** 359–369.
29. Sheetz, M. P., Felsenfeld, D. P., and Galbraith, C. G. (1996) Cell migration: Regulation of force on extracellular-matrix-integrin complexes. *Trends Cell. Biol.* **8,** 51–54.
30. Ponti, A., Machacek, M., Gupton, S. L., Waterman-Storer, C. M., and Danuser, G. (2004) Two distinct actin networks drive the protrusion of migrating cells. *Science* **305,** 1782–1786.
31. Mullins, R. D., Hauser, J. A., and Pollard, T. D. (1988) The interaction of Arp2/3 complex with actin: nucleation, high affinity pointed end capping, and formation of branching network of filaments. *Proc. Natl. Acad. Sci. USA* **95,** 6181–6186.
32. Ghosh, M., Song, X., Mouneimne, G., Sidani, M., Lawrence, D. S., and Condeelis, J. S. (2004) Cofilin promotes actin polymerization and defines the direction of cell motility. *Science* **304,** 743–746.
33. Singer, R., Sagiv, M., Levinsky, H., Maayan, R., Segenreich, E., and Allaouf, D. (1989) The influence of seminal plasma and polyaminic substances on the motility of isolated human sperm. *Int. J. Fertil.* **34,** 224–230.
34. Manni, A. and Wright, C. (1984) Polyamines as mediators of estrogen action on the growth of experimental breast cancer in rats. *J. Natl. Cancer Inst.* **73,** 511–514.
35. Santos, M. F., Viar, M. J., McCormack, S. A., and Johnson, L. R. (1997) Polyamines are important for attachment of IEC-6 cells to extracellular matrix. *Am. J. Physiol. Gastrointest. Liver Physiol.* **273,** G175–G183.
36. McCormack, S. A., Viar, M. J., and Johnson, L. R. (1992) Migration of IEC-6 cells: A model for mucosal healing. *Am. J. Physiol.* **263,** G426–G435.
37. Quaroni, A., Wands, J., Trelstad, R. L., and Isselbacher, K. J. (1979) Epithelial cell cultures from rat small intestine. *J. Cell Biol.* **80,** 248–265.
38. Ray, R. M., McCormack, S. A., Covington, C., Viar, M. J., Zheng, Y., and Johnson, L. R. (2003) The requirement for polyamines for intestinal epithelial cell migration is mediated through Rac1. *J. Biol. Chem.* **278,** 13,039–13,046.
39. McCormack, S. A., Viar, M. J., and Johnson, L. R. (1993) Polyamines are necessary for cell migration by a small intestinal crypt cell line. *Am. J. Physiol.* **264,** G367–G374.
40. Yuan, Q., Viar, M. J., Ray, R. M., and Johnson, L. R. (2000) Putrescine does not support the migration and growth of IEC-6 cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* **278,** G49–G56.
41. McCormack, S. A., Ray, R. M., Blanner, P. M., and Johnson, L. R. (1999) Polyamine depletion alters the relationship of F-actin, G-actin, and thymosine β4 in migrating IEC-6 cells. *Am. J. Physiol.* **276,** C459–C468.
42. Geiger, B., Bershadsky, A., Pankov, R., and Yamada, K. M. (2001) Transmembrane crosstalk between the extracellular matrix-cytoskeleton crosstalk. *Nat. Rev. Mol. Cell Biol.* **2,** 793–805.
43. Emsley, J., Knight, C. G., Farndale, R. W., Barnes, M. J., and Liddington, R. C. (2000) Structural basis of collagen recognition by integrin alpha2beta1. *Cell* **101,** 47–56.
44. Ray, R. M., Viar, M. J., McCormack, S. A., and Johnson, L. R. (2001) Focal adhesion kinase signaling is decreased in polyamine-depleted IEC-6 cells. *Am. J. Physiol. Cell Physiol.* **281,** C475–C485.
45. Hall, A. (1998) Rho GTPases and the actin cytoskeleton. *Science* **279,** 509–514.
46. Chant, J. and Stowers, L. (1995) GTPases cascades choreographing cellular behavior: movement and morphogenesis. *Cell* **81,** 1–4.
47. Nobes, C. D. (2000) Rho GTPases and cell migration-fibroblast wound healing. *Methods Enzymol*. **325,** 441–449.

48. Ridley, A. J. and Hall, A. (1992) The small GTP-binding protein Rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* **70,** 389–399.
49. Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D., and Hall, A. (1992) The small GTP-binding protein Rac regulates growth factor-induced membrane ruffling. *Cell* **70,** 401–410.
50. Nobes, D. and Hall, A. (1995) Rho, Rac, and Cdc42 GTPases regulate the assembly of multi-molecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* **81,** 53–62.
51. Pohjanpelto, P., Virtanen, I., and Holta, E. (1981) Polyamine starvation causes the disappearance of actin filaments and microtubules in polyamine-auxotrophic CHO cells. *Nature* **293,** 475–477.
52. Kaminska, B., Kazmareck, L., and Grzelakowska, B. (1992) Inhibitors of polyamine affect the expression of genes encoding cytoskeletal proteins. *FEBS Lett.* **304,** 198–200.
53. Grant, N. J. and Orial-Audit, C. (1983) Influence of the polyamine spermine on the organization of cortical filaments in isolated cortices of *Xenopus leavis* eggs. *Eur. J. Cell Biol.* **30,** 67–73.
54. Santos, M. F., McCormack, S. A., Zheng, G., et al. (1997) Rho proteins play a critical role in cell migration during the early phase of mucosal restitution. *J. Clin. Invest.* **100,** 216–225.
55. Ray, R. M., Patel, A., Viar, M. J., et al. (2002) RhoA inactivation inhibits cell migration but does not mediate the effects of polyamine depletion. *Gastroenterol.* **1213,** 196–205.
56. Brzozowski, T., Konturek, S. J., Majka, J., Dembinski, A., and Drozdowicz, D. (1993) Epidermal growth factor, polyamines and prostaglandins in healing of stress-induced gastric lesion in rats. *Dig. Dis. Sci.* **38,** 276–283.
57. Riegler, M., Sedivy, R., Sogukoglu, T., et al. (1996) Epidermal growth factor promotes rapid response to epithelial injury in rabbit duodenum *in vitro*. *Gastroenterol.* **111,** 28–36.
58. Furukawa, O., Matsu, H., Suzuki, N., and Okabe, T. (1999) Epidermal growth factor protects rat epithelial cells against acid-induced damage through the activation of the Na^+/H^+ exchangers. *J. Pharmacol. Exp Ther.* **288,** 620–626.
59. Olsen, P. S., Poulsen, S. S., Kirkegaard, P., and Nexo, E. (1984) Role of submandibular saliva and epidermal growth factor in gastric cytoprotection. *Gastroenterol.* **87,** 103–108.
60. Wakabayashi, S., Sardet, C., Fafournoux, P., et al. (1992) Structure function of the growth factor-activatable Na^+/H^+ exchanger (NHE1). *Rev. Physiol. Biochem. Pharmacol.* **119,** 157–186.
61. Thompson, J. F. (1988) Specific receptors for epidermal growth factor in rat intestinal microvillus membranes. *Am. J. Physiol. Gastrointest. Liver Physiol.* **254,** G429–G435.
62. Playford, R. J., Hanby, A. M., Gshmeissner, S., Peiffer, L. P., Wright, N. A., and McGarrity, T. (1999) The epidermal growth factor receptor (EGF-R) is present on the basolateral but not the apical surface of enterocytes in the human gastrointestinal tract. *Gut* **139,** 262.
63. Konturek, P. C., Ernst, H., Brozozowski, T., Ihm, A., and Konturek, S. J. (1997) Expression of epidermal growth factor and transforming growth factor-$\propto$ after exposure of rat gastric mucosa to stress. *Scand. J. Gastroenterol.* **32,** 980–984.
64. Wright, N. A., Pike, C., and Elia, G. (1990) Induction of novel epidermal growth factor-secreting cell lineage by mucosal ulceration in human gastrointestinal stem cells. *Nature* **343,** 82–85.
65. Vaidya, R. J., Ray, R. M., and Johnson, L. R. (2005) MEK1 restores migration of polyamine-depleted cells by retention and activation of Rac1 in the cytoplasm. *Am. J. Physiol. Cell Physiol.* **288,** C350–C359.
66. Xie, H., Pallero M. A., Gupta, K., et al. (1998) EGF receptor regulation of cell motility: EGF induces disassembly of focal adhesions independently of the motility-associated PLCgamma signaling pathway. *J. Cell Sci.* **111,** 615–624.

67. Klemke, R. L., Cai, S., Giannini, A. L., Gallagher, P. J., de Lanerolle, P., and Cheresh, D. A. (1997) Regulation of cell motility by mitogen-activated protein kinase. *J. Cell Biol.* **37,** 481–492.
68. Van Puijenbroek, A. A. F. L., van Weering, D. H. J., van den Brink, C. E., et al. (1997) Cell scattering of SK-N-MC neuroepithelioma cells in response to Ret and FGF receptor tyrosine kinase activation is correlated with sustained ERK2 activation. *Oncogene* **14,** 1147–1157.
69. McCawley, L. J., Li, S., Wattenberg, E. V., and Hudson, L. G. (1999) Sustained activation of the mitogen-activated protein kinase pathway. *J. Biol. Chem.* **274,** 4347–4353.
70. Slack, J. K., Catling, A. D., Eblen, S. T., Weber, M. J., and Parson, J. T. (1999) c-Raf-mediated inhibition of epidermal growth factor-stimulated cell migration. *J. Biol. Chem.* **274,** 27,177–27,184.
71. Guo, H., Ray, R. M., and Johnson, L. R. (2003) RhoA stimulates IEC-6 cell proliferation by increasing polyamine-dependent Cdk2 activity. *Am. J. Physiol. Gastrointest. Liver Physiol.* **285,** G704–G713.
72. Lehman, W., Hatch, V., Korman, V., et al. (2000) Tropomyosin and actin isoforms modulate the localization of tropomyosin strands on actin filaments. *J. Mol. Biol.* **302,** 593–606.
73. McCormack, S. A., Wang, J.-Y., and Johnson, L. R. (1994) Polyamine deficiency causes reorganization of F-actin and tropomyosin in IEC-6 cells. *Am. J. Physiol.* **267,** C715–C722.
74. Wang, J.-Y., Wang, J., Golovina, V. A., Li, L., Platoshyn, O., and Yuan, J. X.-J. (2000) Role of K^+ channel expression in polyamine dependent intestinal epithelial cell migration. *Am. J. Physiol. Cell Physiol.* **278,** C303–C314.
75. Rao, J. N., Li, L., Golovina, V. A., et al. (2001) Ca^{2+}-RhoA signaling pathway required for polyamine-dependent intestinal epithelial cell migration. *Am. J. Physiol. Cell Physiol.* **280,** C993–C1007.
76. Lotz, M. M., Wang, H., Song, J. C., Pories, S. E., and Matthews, J. B. (2004) K+ channel inhibition accelerates intestinal epithelial cell wound healing. *Wound Repair Regen.* **12,** 565–574.
77. Oliver, D., Baukrowitz, T., and Fakler, B. (2000) Polyamines as gating molecules of inward-rectifier K+ channels. *Eur. J. Biochem.* **267,** 5824–5829.
78. Walker, D. C., Hill, G., Wood, S. M., Smallwood, R. H., and Southgate, J. (2004) Agent-based computational modeling of wounded epithelial cell monolayer. *IEEE Trans. Nanobiosci.* **3,** 153–163.
79. Demaurex, N. and Distelhorst, C. (2003) Apoptosis—the calcium connection. *Science* **300,** 65–67.
80. Hajnoczky, G., Davies, E., and Madesh, M. (2003) Calcium signaling and apoptosis. *Biochem. Biophys. Res Commun.* **304,** 445–454.

21

Regulation of Kv Channel Activity and Intercellular Junctions by Polyamines in Intestinal Epithelial Cells

Jaladanki N. Rao and Jian-Ying Wang

1. Introduction

Epithelial cells line the gastrointestinal (GI) mucosa and form an important barrier to a wide array of noxious substances in the lumen. Disruption of this barrier function occurs commonly in various critical illnesses, such as trauma, burns, hemorrhage, sepsis, and massive surgical operations, leading to the translocation of luminal toxic substances and bacteria to the blood stream. Maintenance of the GI epithelial integrity is a complex process that is critically controlled by numerous factors at different levels, but the exact mechanisms involved in this process are far from clear. Over the past decade, it has been shown that polyamines are necessary for normal integrity of the GI epithelium under physiological and various pathological conditions. Polyamines are required for GI epithelial restitution after injury and also implicated in expression of intercellular junction proteins. Increasing evidence indicates that polyamines regulate rapid epithelial restitution and intercellular junction expression in association with their ability to modulate activity of voltage-gated K^+ channels (Kv) and cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$). In this chapter, we will provide an overview of regulatory effects of cellular polyamines on Kv channels and intercellular junctions in intestinal epithelial cells.

2. Polyamines Modulate Kv Channels Activity and Ca^{2+} Homeostasis

2.1. Polyamines and Kv Channel Expression

Polyamines are ubiquitous organic cations of low molecular weight found in all eukaryotic cells and are known to be intimately involved in a variety of epithelial cell functions, including migration, cell-to-cell interaction, proliferation, and apoptosis *(1–6)*. Cellular polyamine levels are highly regulated by biosynthesis, degradation, and transport system. Polyamines synthesis depends on the activation or inhibition of ornithine decarboxylase (ODC), which catalyzes the first rate-limiting step in polyamine

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biosynthesis. ODC decarboxylates the amino acid ornithine to form putrescine (1,4-diaminobutane); propylamine groups are then added to one or both amino groups of putrescine to form spermidine and spermine, respectively. Another enzyme involved in this is *S*-adenosylmethionine decarboxylase, and it may also be rate-limiting. Instead of conversion into spermidine, putrescine can be degraded by diamine oxidase or monoamine oxidase. It has been demonstrated that spermidine/spermine N^1-acetyltransferase is a rate-limiting factor in the polyamine degradation *(1)*.

Kv channels are a group of membrane proteins and play an important role in regulating resting membrane potential (E_m) in many types of cells *(7,8)*. At the molecular level, Kv channels are composed of the pore-forming α subunits and the regulatory β subunits *(7,9,10)*. Activation of Kv channels causes membrane hyperpolarization, whereas inhibition of Kv channels predisposes to membrane depolarization. A series of studies from our laboratory has recently demonstrated that intracellular polyamines are absolutely required for the expression of Kv channels in normal intestinal epithelial cells *(5,11)*. Depletion of cellular polyamines inhibits expression of Kv channel α subunits (Kv1.1), reduced whole-cell K^+ currents ($I_{K(v)}$), and depolarized E_m (Fig. 1). The subsequent membrane depolarization decreases the Ca^{2+} driving force and thus inhibits Ca^{2+} influx. Polyamine depletion does not alter expression of the Kv channel β subunit. In contrast, increased cellular polyamines by addition of exogenous spermidine or by overexpression of the *ODC* gene stimulate Kv channel activity and result in membrane hyperpolarization. These results clearly indicate that polyamines are major stimulators for expression of the Kv channel genes and are involved in the control of E_m in intestinal epithelial cells.

2.2. Kv Channel Activity and Ca^{2+} Homeostasis in Intestinal Epithelial Cells

Increasing evidence shows that activity of Kv channels controls E_m that regulates $[Ca^{2+}]_{cyt}$ concentration by governing the driving force for Ca^{2+} influx *(5,9)*. At the cellular level, $[Ca^{2+}]_{cyt}$ is derived from two sources—external and internal stores *(12–14)*. Ca^{2+} can enter from outside the cell by passing through channels that span the external barrier, plasma membrane, and also be released from internal Ca^{2+} stores (endoplasmic or sarcoplasmic reticuli). Ca^{2+} influx depends on the Ca^{2+} driving force, or the electrochemical gradient across the plasma membrane. Although the chemical gradient, the ratio of extracellular $[Ca^{2+}]$ ($[Ca^{2+}]_o$) to $[Ca^{2+}]_{cyt}$, and the Ca^{2+} equilibrium potential, E_{Ca} {$E_{Ca} = 12.5 \ln ([Ca^{2+}]_o/[Ca^{2+}]_{cyt}) = 117–131$ mV at 25°C} are constant, the Ca^{2+} driving force is mainly determined by the electrical gradient, the difference between E_m and E_{Ca} ($E_m - E_{Ca}$). In other words, E_m is a major determinant of the driving force for Ca^{2+} influx.

By controlling the Ca^{2+} driving force, E_m is an important regulator of $[Ca^{2+}]_{cyt}$ in nonexcitable cells including epithelial cells and lymphocytes. Membrane depolarization, such as after polyamine depletion, decreases the Ca^{2+} driving force and inhibits Ca^{2+} influx. In contrast, membrane hyperpolarization after increased polyamines increases the Ca^{2+} driving force and enhances Ca^{2+} influx. Therefore, in the cells that do not express L-type voltage-dependent Ca^{2+} channels (VDCC), Ca^{2+} influx is decreased by membrane depolarization but increased by membrane hyperpolarization.

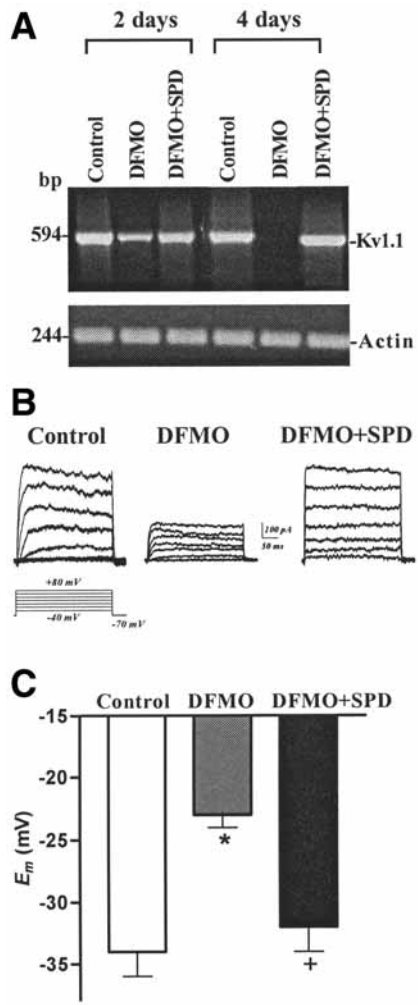

Fig. 1. Effect of depletion of cellular polyamines by α-difluoromethylornithine (DFMO) on mRNA expression of voltage-gated K^+ (Kv)1.1 channel **(A)**, whole cell Kv currents **(B)**, and resting membrane potential **(C)** in intestinal epithelial cells (IEC-6 line). Cells were grown in the Dulbecco's modified Eagle's medium containing 5% dialyzed fetal bovine serum and 5 m*M* DFMO with or without 5 μ*M* spermidine (SPD) for 4 d. *, + $p < 0.05$ compared with control and DFMO alone, respectively.

Nonetheless, in excitable cells such as neurons, cardiomyocytes, and muscle cells, VDCC that are opened by membrane depolarization are the major pathway for Ca^{2+} influx *(7)*. In contrast to the voltage-independent pathway for Ca^{2+} influx in non-excitable cells, membrane depolarization opens VDCC and thus increases $[Ca^{2+}]_{cyt}$ in excitable cells.

E_m is primarily determined by the K^+ permeability and K^+ gradient across the plasma membrane *(7)*. Because the K^+ gradient is maintained by Na^+-K^+ adenosine triphosphatase, the K^+ permeability is directly related to the activity and number of membrane K^+ channels. Kv channels are a major determinant of resting E_m in many types of cells. When K^+ channels close or the total numbers of total K^+ channels decrease, E_m becomes less negative (i.e., depolarization). When K^+ channels open or the numbers of total K^+ channels rise, E_m becomes more negative (i.e., hyperpolarization) *(7,9)*. Therefore, inhibition of K^+ channel gene expression in polyamine-deficient cells would decrease the number of K^+ channels and attenuate K^+ channel activity. The subsequent membrane depolarization decreases the Ca^{2+} driving force, and thus inhibits Ca^{2+} influx. Because Ca^{2+} entry is a major source for $[Ca^{2+}]_{cyt}$, inhibition of Ca^{2+} influx would reduce $[Ca^{2+}]_{cyt}$ in cells lacking VDCC *(7)*.

2.3. $[Ca^{2+}]_{cyt}$ Through CCE and Transient Receptor Potential Channels

Polyamine-modulated Ca^{2+} driving force is critical for transmembrane influx of Ca^{2+} in intestinal epithelial cells, but specific Ca^{2+}-permeable channels in this process are still unknown. In nonexcitable cells, passive Ca^{2+} leakage, receptor-operated Ca^{2+} channels, nonselective cation channels, and store-operated channels, all contribute to Ca^{2+} influx *(13,14)*. It has been shown that intestinal epithelial cells lack VDCCs, but have developed the Ca^{2+} entry mechanism that is coupled with the depletion of intracellular Ca^{2+} stores *(5,11,15)*. Thus the capacitative Ca^{2+} entry (CCE) is important in maintaining sustained Ca^{2+} influx and in refilling Ca^{2+} into the stores, both of which are required for cell migration *(5,16)*.

The molecular basis for the activation and regulation of CCE has not yet been determined in intestinal epithelial cells and any other nonexcitable cell types. The two critical questions that remain are the molecule that mediates Ca^{2+} influx into the cells and the signal that is transmitted from the internal Ca^{2+} store to the plasma membrane to trigger activation or inactivation of CCE. Studies toward identifying the molecules mediating Ca^{2+} influx have led to the cloning of mammalian homologs of the *Drosophila* transient receptor potential genes, *TRP (17)*. To date, seven genes encoding TRPC channels, a subgroup of the *TRP* channels, have been cloned in mammalian species, including human, mouse, rat, rabbit, and bovine *(18,19)*. The products of these genes are proposed to encode the CCE channels in nonexcitable cells. Expression of *TRPC* genes in *Xenopus oocytes* or mammalian cells results in the formation of Ca^{2+}-permeable cation channels, which are activated by Ca^{2+} store depletion.

We have recently demonstrated that normal intestinal epithelial cells (IEC-6 line) expressed TRPC1 and TPRC5 and displayed typical records of I_{soc} and CCE generated by Ca^{2+} influx after depletion of intracellular stores. Forced expression of the *TRPC1* gene in stable TRPC1-transfected IEC-6 cells increased $[Ca^{2+}]_{cyt}$ through CCE. Differentiated IEC-Cdx2L1 cells induced by forced expression of the *Cdx2* gene highly expressed endogenous TRPC1 and TRPC5 and exhibited increased CCE after Ca^{2+} store depletion. Specific inhibition of endogenous TRPC1 expression by transfection with the TRPC1 small interfering RNA decreased $[Ca^{2+}]_{cyt}$ by reducing CCE in the Ca^{2+} store-depleted cells. These findings strongly suggest that TRPC1 functions as the

store-operated Ca^{2+} channels in intestinal epithelial restitution and is implicated in Ca^{2+} homeostasis through CCE.

2.4. $[Ca^{2+}]_{cyt}$ and Polyamine-Dependent Cell Migration

GI mucosal injury occurs commonly from mild physical trauma during digestion, critical and chronic illnesses, various surgical conditions, the ingestion of alcohol, aspirin, nonsteroidal anti-inflammatory compounds, or *Helicobacter pylori* infection. Restoration of normal intestinal mucosal integrity—successful repair of wounds and ulcers—requires epithelial cell decisions that regulate signaling networks controlling gene expression, survival, migration, and proliferation. In the acute response to injury, damaged cells are sloughed, and remaining viable cells from areas adjacent to or just beneath the injured surface migrate to cover the denuded area. This early restitution refers to resealing of superficial wounds as a consequence of epithelial cell migration into the defect, a process independent from epithelial cell proliferation *(3,20)*. This rapid repair appears to be an initial host response to prevent noxious agents from causing deeper tissue damage. The other repair process is the replacement of lost cells by cell proliferation and is much slower *(21,22)*. Rapid epithelial restitution is regulated by numerous factors, including cellular polyamines. Cellular polyamine levels are dramatically increased during the process of early mucosal restitution in both in vivo and in vitro systems, and polyamine depletion through inhibition of ODC inhibits cell migration and delays mucosal restitution.

We have demonstrated that polyamines regulate intestinal epithelial cell migration by altering K^+ channel activity, E_m, and $[Ca^{2+}]_{cyt}$ and that the resultant increase in $[Ca^{2+}]_{cyt}$ exerts its regulatory effects on cell motility through interaction with specific targets during restitution *(5,6,11,15)*. Because intestinal epithelial cells do not express VDCC, the depolarized E_m by polyamine depletion decreases $[Ca^{2+}]_{cyt}$ through the reduced driving force for Ca^{2+} influx (Fig. 2). Migration is reduced by 80% in the polyamine-deficient cells. Decreased $[Ca^{2+}]_{cyt}$ by depolarization of E_m by 4-aminopyridine also inhibits normal cell migration and prevents the restoration of cell migration by exogenous spermidine in polyamine-deficient cells. In contrast, increased $[Ca^{2+}]_{cyt}$ by the treatment with Ca^{2+} ionophore ionomycin stimulates cell migration in the absence of cellular polyamines (Fig. 2). These results indicate that polyamine-mediated intestinal epithelial cell migration is due partially to increase of Kv channel expression. The subsequent membrane hyperpolarization after increased levels of polyamines raises $[Ca^{2+}]_{cyt}$ by increasing the driving force for Ca^{2+} influx and, thus, stimulates cell migration.

In another set of experiments we have further revealed that differentiated IEC-Cdx2L1 cells migrate over the wounded edge much faster than undifferentiated parental IEC-6 cells and that increased migration of differentiated IEC-Cdx2L1 cells after wounding results, at least partially, from the K^+ channel activation and the increase in driving force for Ca^{2+} influx during restitution *(11,20)*. Differentiated IEC-Cdx2L1 cells express higher basal levels of Kv1.1 and Kv1.5 mRNAs and proteins than those observed in undifferentiated parental IEC-6 cells. Depletion of intracellular polyamines significantly decreases the expression of both Kv1.1 and Kv1.5 channel genes. Neither

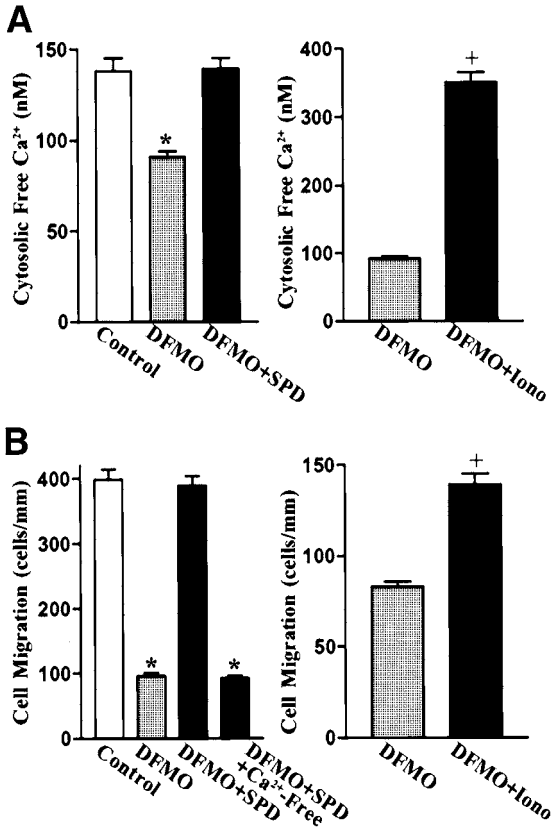

Fig. 2. Effects of polyamine depletion and the Ca^{2+} ionophore ionomycin (Iono) on cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) and cell migration after wounding. **(A)** Summarized data showing $[Ca^{2+}]_{cyt}$ measured in peripheral areas of cells that were grown in control cultures and in cultures containing 5 m*M* α-difluoromethylornithine (DFMO) with or without 5 μ*M* spermidine for 4 d, and cells treated with DFMO for 4 d and then exposed to Iono (1 μ*M*). **(B)** Summarized data showing cell migration after wounding in cells described in **(A)**. Polyamine depletion reduced $[Ca^{2+}]_{cyt}$ and inhibited cell migration (left), whereas increased $[Ca^{2+}]_{cyt}$ by Iono stimulated cell migration in polyamine-deficient cells (right). *, + $p < 0.05$ compared with controls and DFMO alone.

IEC-Cdx2L1 cells nor parental IEC-6 cells express VDCC. The increased expression of Kv channels in differentiated IEC-Cdx2L1 cells is associated with an increase in whole cell K^+ currents, membrane hyperpolarization, and rise of resting $[Ca^{2+}]_{cyt}$. The migration rates in differentiated IEC-Cdx2L1 cells are approx four times of parental IEC-6 cells. Inhibition of Kv channel expression by depletion of cellular polyamines reduced $[Ca^{2+}]_{cyt}$, resulted in cellular reorganization of cytoskeletal proteins, along with a marked reduction in actomyosin stress fiber formation, and inhibited epithelial cell migration. In contrast, elevation of $[Ca^{2+}]_{cyt}$ by the Ca^{2+} ionophore, ionomycin,

promoted formation of actomyosin stress fibers, and increased epithelial cell migration after wounding.

2.5. β-Catenin Tyrosine Phosphorylation and Polyamine-Dependent Restitution

Normal intestinal epithelial cells are tightly bound in sheets, but they need to rapidly disassemble during restitution. A key element in the regulation of cell–cell contacts is the tyrosine phosphorylation of the protein that is localized in the intercellular junctions *(23)*. Among these proteins, β-catenin is considered to be a tyrosine phosphorylation-sensitive component of the adhesion complexes. The activation of β-catenin disassociates E-cadherin from cytoskeleton that causes disassembly of adhesion junctions, enhancing the spreading of cells. Tyrosine phosphorylation of β-catenin reduces its interaction with α-catenin and this reduced interaction may lead to an overall decrease in intercellular contacts, thus promoting cell disassembly and consequent spreading during restitution.

To determine the mechanism by which polyamine-modulated Ca^{2+} induces cell migration during restitution, we have demonstrated that migration of intestinal epithelial cells after wounding is associated with a significant increase in β-catenin tyrosine phosphorylation, which decreases the binding activity of β-catenin to α-catenin *(24)*. Decreased levels of cellular polyamines after treatment with DFMO inhibit Kv channel expression, reduce $[Ca^{2+}]_{cyt}$, prevent the induction of β-catenin phosphorylation, and decrease cell migration. Elevation of $[Ca^{2+}]_{cyt}$ restores β-catenin phosphorylation and promotes migration in polyamine-deficient cells. Decreased β-catenin phosphorylation by tyrosine kinase inhibitors blocks cell migration. These results indicate that β-catenin tyrosine phosphorylation plays a critical role in polyamine-dependent cell migration and that polyamines induce β-catenin tyrosine phosphorylation at least partially through $[Ca^{2+}]_{cyt}$.

3. Polyamines are Necessary for Expression of Intercellular Junction Proteins

3.1. Intercellular Junctions and Epithelial Barrier

Integrity of the intestinal barrier depends on a complex of proteins composing different intercellular junctions, which include tight junctions, adherens junctions, and desmosomes. Over the past several years, our group and others have demonstrated that polyamines are necessary for expression and functions of intercellular junction proteins, especially adherens junctions, and play a critical role in maintenance of intestinal epithelial integrity *(24–26)*. The tight junction is the most apical of these junctional complexes and it seals epithelial cells together in a way that prevents even small molecules from leaking between cells. Immediately below the tight junctions are the cadherin-rich adherens junctions that mediate strong cell-to-cell adhesion and have functional role in forming and regulating the epithelial barrier *(27–29)*. Increasing evidence indicates that adherens junctions integrate various intracellular and extracellular signals and are involved in control of the intestinal epithelial barrier function *(25,30,31)*. Inhibition of the cadherin-dependent adherens junctions by the specific

antibody against E-cadherin or ectopic expression of an E-cadherin dominant–negative mutant retards the assembly of tight junctions and disrupts epithelial barrier function *(32,33)*. Furthermore, adherens junctions are shown to be more vulnerable than tight junctions in some clinical conditions and are of significance in the pathogenesis of barrier failure.

E-cadherin is primarily found at the adherens junctions and is the major cadherin expressed in epithelial cells, including those of the GI tract. As a classical member of adhesion molecules, E-cadherin is a single transmembrane protein containing an extracellular domain with Ca^{2+}-binding sequence repeat motifs and a C-terminal cytoplasmic domain *(32–34)*. E-cadherin dimers bind homotypically to similar dimers on neighboring cells, and its conserved cytoplasmic domain interacts with the cytoskeleton through actin-binding proteins, leading to strengthened cell–cell adhesion *(35)*. The strong E-cadherin-mediated cell–cell adhesions are thought to be necessary for epithelial barrier function, and signals that are transmitted through the adherens junctions may also regulate tight junctions. Increasing evidence suggests that the assembly of tight junctions between epithelial cells requires the prior formation of adherens junctions and that the cadherin-mediated adherens junctions bring two plasma membranes together physically, and permit or enhances tight junction assembly.

In support of this possibility, inhibition of adherens junctions by either treatment with the specific antibody against the extracellular domain of E-cadherin or ectopic expression of a dominant–negative mutant E-cadherin retards the assembly of tight junctions and disrupts epithelial paracellular barrier function *(34,35)*. In contrast, stimulation of E-cadherin expression by exposure of gastric epithelium to a low pH environment enhances adherens junction formation and stabilizes epithelial barrier against acid back-diffusion *(36)*. Consistent with observations in epithelial cells, overexpression of a mutated nonfunctional N-cadherin inhibits endothelial adhesions, impairs tight junction assembly, and decreases vascular endothelial barrier function *(37)*. More recently, it has been reported that overexpression of a mutant VE-cadherin lacking an adhesive extracellular domain disrupts endothelial barrier function and also inhibits endothelial growth in dermal microvascular endothelial cells *(33)*. These findings strongly suggest that the adherens junctions are able to integrate a variety of extracellular and intracellular signals to regulate epithelial barrier function.

3.2. Polyamines and E-Cadherin Expression

A series of studies from our laboratory has demonstrated that polyamines are necessary for expression of E-cadherin in intestinal epithelial cells and that polyamine-modulated E-cadherin plays an important role in maintenance of normal epithelial barrier functions *(24–26)*. To determine the involvement of polyamines in regulation of cell–cell adherens junctions and tight junctions, normal intestinal epithelial cells (IEC-6 line) were grown in the presence or absence of α-difluoromethylornithine (DFMO), the highly specific inhibitor of polyamine synthesis. Treatment with DFMO completely depleted putrescine within 48 h, but it took 4 d to totally deplete spermidine and significantly decrease spermine (by >60%). Similar results have been published previously *(38,39)*.

Depletion of cellular polyamines by DFMO selectively and significantly inhibited mRNA expression of E-cadherin, but negligibly affected levels of other adherens proteins such as β-catenin and α-catenin mRNAs. The levels of E-cadherin mRNA in the cells treated with DFMO for 4, 6, and 8 d were decreased by approx 60%. The changes in E-cadherin mRNA were paralleled by those of E-cadherin proteins (Fig. 3). The levels of E-cadherin protein in the cells exposed to DFMO for 4, 6, and 8 d were decreased by approx 80%. Although there was no significant inhibition of β-catenin and α-catenin mRNA expression in DFMO-treated cells, protein levels of these two adherens junction proteins decreased significantly at 6 and 8 d after polyamine depletion (Fig. 3A). The levels of β-catenin and α-catenin proteins were decreased by approx 20 and approx 15%, respectively. In the presence of DFMO, decreased expression of E-cadherin mRNA and protein, β-catenin, and α-catenin proteins was completely prevented by addition of exogenous spermidine (5 μ*M*). We also examined the effect of polyamine depletion on E-cadherin expression in Caco-2 cells and demonstrated that changes in E-cadherin levels were similar to those observed in IEC-6 cells. Immunofluorescence staining was performed to determine cellular distribution of E-cadherin and β-catenin in IEC-6 cells. In control cells (Fig. 3B), immunoreactivities for E-cadherin and β-catenin were mainly located along the entire cell–cell contact region of adjacent cells. In DFMO-treated cells, these membrane immunoreactivities for E-cadherin and β-catenin markedly were decreased. Exogenous spermidine given together with DFMO reversed the inhibitory effect of polyamine depletion on E-cadherin and β-catenin formation. These results clearly indicate that polyamines are necessary for expression of E-cadherin protein in intestinal epithelial cells.

To test the possibility that the decrease in E-cadherin mRNA level in polyamine-deficient cells results from inhibition of the gene transcription, we examined changes in E-cadherin promoter activity by using luciferase reporter gene assays *(40)*. Consistent with its effect on E-cadherin mRNA expression, polyamine depletion by DFMO decreased E-cadherin promoter activity dramatically. The activity of E-cadherin promoter luciferase reporter was decreased by approx 70% on d 6 after administration of DFMO, which was completely prevented by spermidine given together with DFMO. We also examined changes in E-cadherin protein stability after polyamine depletion by measurement of the half-life of E-cadherin protein by pulse-chase analysis. In control cells, E-cadherin protein decreased at a relatively slow rate, with about 60% remaining after 6 h. On the other hand, levels of E-cadherin protein in polyamine-deficient cells decreased quickly, with a half-life of approx 4 h, indicating that E-cadherin protein degradation was approx 20% faster after polyamine depletion. Changes in E-cadherin mRNA stability and its translation rate were also examined in the presence or absence of polyamines. The stability and translation of E-cadherin mRNA in IEC-6 cells was not affected by polyamine depletion. There were no significant changes in E-cadherin mRNA half-life and its translation rate between control cells and cells exposed to DFMO with or without spermidine for 6 d. Taken together, these findings indicate that polyamines predominantly regulate transcription of the *E-cadherin* gene, although they also slightly modulate E-cadherin protein stability in intestinal epithelial cells.

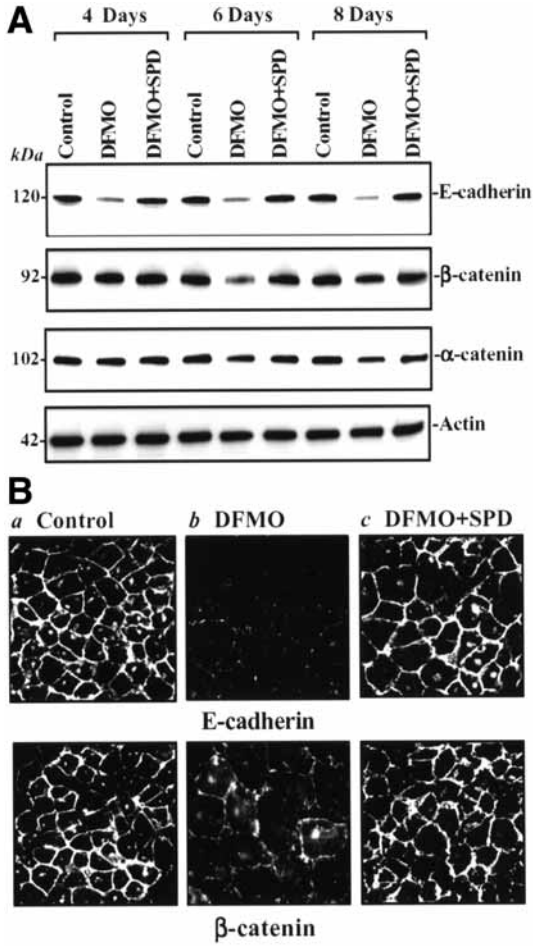

Fig. 3. Changes in protein levels of E-cadherin, β-catenin, and α-catenin after polyamine depletion in IEC-6 cells. **(A)** Representative autoradiograms of Western blots. E-cadherin (~120 kDa), β-catenin (~92 kDa), and α-catenin (~102 kDa) were identified by probing nitrocellulose with specific antibodies. **(B)** Cellular distribution of E-cadherin and β-catenin: *a*, control; *b*, cells treated with α-difluoromethylornithine (DFMO) alone for 6 d; and *c*, cells treated with DFMO + spermidine for 6 d. Cells were permeabilized and incubated with the specific antibody against E-cadherin or β-catenin and then with anti–immunoglobulin G conjugated with FITC. Original magnification: ×1000.

3.3. Intracellular Ca^{2+} and Polyamine-Dependent E-Cadherin Expression

Polyamines modulate $[Ca^{2+}]_{cyt}$ by governing the driving force for Ca^{2+} influx via controlling activity of Kv channels *(5,11)* and that Ca^{2+} mediates distinct actions of intestinal epithelial cells after increased polyamines *(6)*. Because IEC-6 cells do not express VDCC, the inhibition of Kv channel expression and resultant membrane depolarization through

polyamine depletion decreases the driving force for Ca^{2+} influx and reduces $[Ca^{2+}]_{cyt}$ concentration. To test the possibility that polyamines regulate E-cadherin expression by altering $[Ca^{2+}]_{cyt}$, the following two studies were carried out in IEC cells.

The first study examined whether the decreased $[Ca^{2+}]_{cyt}$ by removal of extracellular calcium altered expression of E-cadherin mRNA and protein in control cells (without DFMO). Removal of extracellular Ca^{2+} not only decreased E-cadherin mRNA, but also inhibited E-cadherin protein expression (Fig. 4A). This inhibitory effect on E-cadherin expression occurred at 4 h and peaked at 6 h after exposure to the Ca^{2+}-free medium *(25)*. Levels of E-cadherin mRNA and protein were decreased by approx 60% at 4 h and approx 90% at 6 h after incubation with the Ca^{2+}-free medium, respectively. Some small protein fragments were regularly detected in cells exposed to the Ca^{2+}-free medium for 4 and 6 h, probably resulting from the stimulation of E-cadherin degradation. The regulatory effect of Ca^{2+} on E-cadherin is specific because expression of β-catenin protein was not affected after removal of extracellular Ca^{2+} *(24)*. There were no significant differences in levels of β-catenin protein between control cells and cells exposed to the Ca^{2+}-free medium for 2, 4, and 6 h.

The second study determined whether increasing $[Ca^{2+}]_{cyt}$ by the Ca^{2+} ionophore ionomycin prevented the inhibition of E-cadherin expression in polyamine-deficient cells. Consistent with our previous findings *(6,11)*, exposure to 1 μ*M* ionomycin dramatically increased $[Ca^{2+}]_{cyt}$ in DFMO-treated cells. Increased $[Ca^{2+}]_{cyt}$ by ionomycin also increased levels of E-cadherin mRNA and protein in the absence of polyamines (Fig. 4B). Treatment of polyamine-deficient cells with ionomycin for 6 h restored E-cadherin mRNA to approx 85% of control value and its protein to approx 70%, respectively. Neither the Ca^{2+}-free medium nor ionomycin affected cell attachment and cell viability in control and DFMO-treated cells as measured by trypan blue staining method. These results indicate that polyamines regulate E-cadherin expression, at least partially, through $[Ca^{2+}]_{cyt}$ in intestinal epithelial cells.

3.4. c-Myc and Polyamine-Induced Transcription of the E-Cadherin *Gene*

The c-Myc protein is a nuclear transcription factor, and its gene expression absolutely requires polyamines *(41,42)*. Recently, *E-cadherin* was identified as a c-Myc target gene and activation of c-Myc enhances *E-cadherin* gene transcription in a cell type-dependent manner *(43)*. Our recent study provides new evidence showing that polyamines regulate transcription of the *E-cadherin* gene through c-Myc in normal intestinal epithelial cells.

Depletion of cellular polyamines dramatically inhibits *c-myc* gene transcription and decreased levels of c-Myc protein *(39,41,42)*, which is associated with a significant reduction of E-cadherin promoter activity in IEC-6 cells *(40)*. We have successfully constructed the adenoviral vector containing *c-Myc* cDNA (AdMyc) under the control of the human cytomegalovirus immediate early gene promoter. Infection of IEC-6 cells with AdMyc vector increased c-Myc protein by approx 20-fold, and this forced expression of the *c-Myc* stimulated transcription of the *E-cadherin* gene as indicated by a significant increase in E-cadherin promoter activity. The level of E-cadherin-promoter activity was increased by approx threefold when cells were infected with the AdMyc at

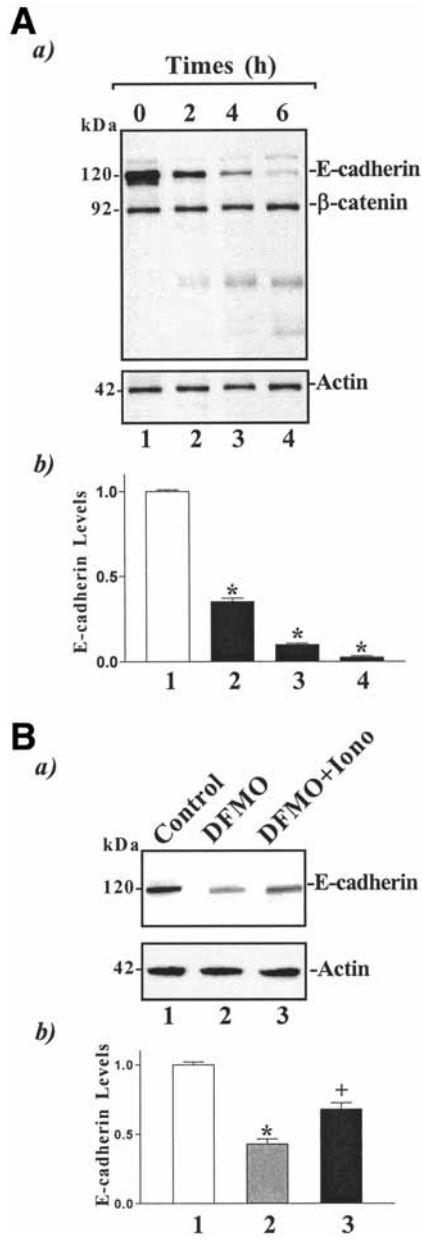

Fig. 4. Effects of manipulating intracellular Ca^{2+} concentration, either decreased by removal of extracellular Ca^{2+} or increased by the Ca^{2+} ionophore ionomycin (Iono), on E-cadherin protein expression. **(A)** Levels of E-cadherin protein in control cells exposed to Ca^{2+}-free medium: *a*, representative autoradiograms of Western blots; *b*, quantitative analysis of Western immunoblots by densitometry from cells described in *a*, Open bar, no exposure to Ca^{2+}-free

the dose of 50 pfu/cell. These results clearly show that activation of c-Myc enhances *E-cadherin* gene expression in intestinal epithelial cells.

3.5. Polyamines Also Regulate Tight Junction Proteins

A recent study has shown that polyamines are required for normal function of tight junctions and those polyamines regulate expression of various tight junction proteins through different mechanisms *(26)*. In this study, differentiated IEC-Cdx2L1 cells were used, because this line of cells has well-developed tight junctions. Depletion of cellular polyamines by DFMO treatment decreased protein levels of tight junctions occludin, ZO-1, ZO-2, claudin-2, and claudin-3 in differentiated IEC-Cdx2L1 cells. The levels of occludin protein in the cells exposed to DFMO for 4, 6, and 8 d were decreased by more than 80% (Fig. 5). Although there was no inhibition of ZO-1 and ZO-2 expression in undifferentiated parental IEC-6 cells treated with DFMO, levels of ZO-1 and ZO-2 proteins in differentiated IEC-Cdx2L1 cells decreased significantly after polyamine depletion *(25)*. The levels of ZO-1 and ZO-2 proteins in differentiated IEC-Cdx2L1 cells exposed to DFMO for 4, 6, and 8 d were decreased by approx 55 and approx 40%, respectively. Treatment with DFMO for 4 d did not alter expression of claudin-2, but its levels were decreased by approx 50% on d 6 and by approx 80% on d 8, respectively (Fig. 5). Changes in claudin-3 expression were similar to those observed in claudin-2 after polyamine depletion. In the presence of DFMO, decreased levels of occludin, ZO-1, ZO-2, claudin-2, and claudin-3 proteins were completely abolished by addition of exogenous spermidine. Interestingly, depletion of cellular polyamines did not inhibit expression of occludin, ZO-1, and ZO-2 mRNAs, although it significantly decreased levels of their proteins. On the other hand, polyamine depletion inhibited expression of claudin-2 and claudin-3 mRNAs, which was completely prevented by exogenous spermidine. These findings suggest that polyamines are involved in expression of various tight junction proteins through different signaling pathways in intestinal epithelial cells.

To determine whether polyamines regulate occludin at the translation level, the level of newly synthesized occludin protein was examined. Polyamine depletion by DFMO significantly decreased the occludin protein synthesis in differentiated IEC-Cdx2L1 cells. The level of newly synthesized occludin protein was decreased by approx 70% in cells exposed to DFMO for 6 d. We also examined changes in occludin protein stability after polyamine depletion by measurement of the half-life of occludin protein by pulse-chase analysis. In control cells (without DFMO), the levels of occludin protein declined gradually after protein synthesis was inhibited by cycloheximide, with a half-life of approx 120 min. On the other hand, levels of occludin protein in polyamine-deficient

medium; filled bars, exposure to Ca^{2+}-free medium. Values are means ± SE; relative E-cadherin protein levels were corrected for loading as measured by densitometry of actin. *$p < 0.05$ vs 0 h group. **(B)** Levels of E-cadherin protein in polyamine-deficient cells exposed to ionomycin for 6 h: *a*, representative autoradiograms of Western blots; *b*, quantitative analysis of Western immunoblots by densitometry from cells described in *a*. Open bars, control; gray bars, DFMO; filled bars, DFMO + Iono. Values are means ± SE. *$p < 0.05$ vs control group; + $p < 0.05$ vs DFMO-treated cells.

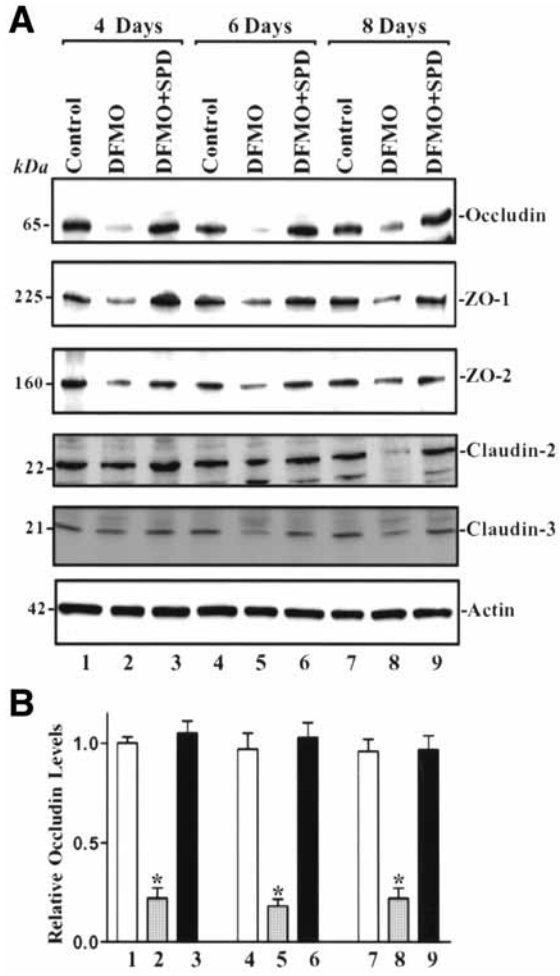

Fig. 5. Changes in expression of tight junction proteins occludin, ZO-1, ZO-2, claudin-2, and claudin-3 in differentiated IEC-Cdx2L1 cells treated with DFMO alone or DFMO + spermidine (SPD). Before experiments, stable IEC-Cdx2L1 cells were grown in DMEM containing 4 m*M* isopropyl β-D-thiogalactopyranoside for 16 d to induce cell differentiation. These differentiated IEC-Cdx2L1 cells were grown in DMEM containing DFMO or DFMO plus SPD for 4, 6, and 8 d. **(A)** Representative immunoblots of Western analysis. **(B)** Quantitative analysis derived from densitometric analysis of immunoblots of occludin from cells described in **(A)**. Values are means ± SE of data from three separate experiments; relative levels of proteins were corrected for loading as measured by densitometry of actin. *$p < 0.05$ compared with the corresponding control and DFMO + SPD.

cells decreased quickly, with a half-life of approx 75 min, indicating that occludin protein degradation was faster after polyamine depletion. Spermidine given together with DFMO not only totally overcame the decrease in occludin protein synthesis, but also

completely prevented the instability of occludin. These findings clearly indicate that polyamines are implicated in regulation of occludin protein synthesis and stability in intestinal epithelial cells.

3.6. Dysfunction of Intestinal Epithelial Barrier After Polyamine Depletion

Polyamine depletion results in dysfunction of the epithelial barrier as indicated by a decrease in transepithelial electrical resistance (TEER) and increase in paracellular permeability *(25,26)*. To study the possible role of decreased intercellular junctions in intestinal epithelial paracellular barrier function, TEER and paracellular flux of membrane impermeable tracers across the confluent monolayer were examined in the presence or absence of polyamines *(25)*. IEC-6 cells were grown in control cultures or cultures containing DFMO or DFMO plus spermidine for 4 d, then plated at confluent density on the insert, and maintained for an additional 48 h to establish a tight monolayer. Two widely accepted hydrophilic paracellular tracer molecules, [^{14}C]-mannitol and [^{3}H]-inulin, were used in this study. To verify the system used for paracellular permeability assays, the effects of decreased $[Ca^{2+}]_{cyt}$ on the paracellular flux of [^{14}C]-mannitol and [^{3}H]-inulin were tested, and served as a positive control. As expected, exposure to the Ca^{2+}-free medium for 2 h markedly increased paracellular permeability in normal cells (without DFMO) (Fig. 6, left). Consistent with inhibitory effect on intercellular junction expression, polyamine depletion also significantly decreased TEER and increased paracellular permeability (Fig. 6, right). In DFMO-treated cells, values of TEER were decreased by approx 35%, whereas levels of paracellular flux of [^{14}C]-mannitol and [^{3}H]-inulin were increased by approx 30 and approx 25%, respectively. Spermidine given together with DFMO restored the paracellular permeability to normal levels. Levels of paracellular permeability in cells exposed to DFMO plus spermidine were similar to those observed in control cells.

Differentiated IEC-Cdx2L1 cells were also used to determine the relationship between decreased expression of intercellular junctions and paracellular permeability after polyamine depletion. These stable IEC-Cdx2L1 cells were polarized, exhibited multiple morphological characteristics of villous-type enterocytes including well-developed tight junctions. Consistent with parental IEC-6 cells, polyamine depletion not only inhibited intercellular junction expression, but also decreased TEER, which was associated with an increase in paracellular permeability in differentiated IEC-Cdx2L1 cells *(25)*. Taken together, these findings indicate that polyamines are essential for integrity of intestinal epithelial barrier function by regulating expression of intercellular junctions.

4. Summary and Conclusions

The results summarized in this chapter clearly indicate that polyamines are implicated in regulation of K^+ channel activity and intracellular Ca^{2+} homeostasis in normal intestinal epithelial cells. Polyamines are potent stimulators for expression of the Kv channel genes, and the resultant increase in Kv channel activity is critical for the control of Ca^{2+} influx by regulating E_m that governs the driving force for Ca^{2+} influx during restitution. Increased levels of cellular polyamines activate K^+ channel activity,

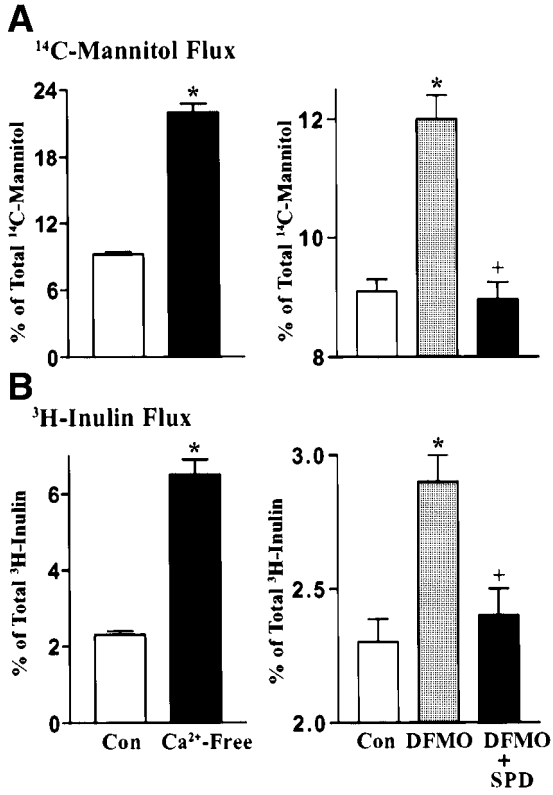

Fig. 6. Changes in paracellular permeability in IEC-6 cells in the presence or absence of cellular polyamines. After cells were grown in control cultures or cultures containing either 5 m*M* DFMO alone or DFMO plus 5 μ*M* spermidine (SPD) for 4 d, they were trypsinized, plated at confluent density on the insert, and then maintained at same culture conditions for additional 48 h. The entire basal medium was collected 2 h after addition of [^{14}C]-mannitol or [^{3}H]-inulin for paracellular tracer flux assays. Values are means ± SE of data from eight samples. *$p < 0.05$ compared with control group; +$p < 0.05$ compared with DFMO-treated cells.

increase $[Ca^{2+}]_{cyt}$ through increases in the driving force for Ca^{2+} influx, and promote cell migration during restitution after injury. In contrast, polyamine depletion decreases epithelial cell migration by reducing $[Ca^{2+}]_{cyt}$ through inactivation of Kv channel activity, thus leading to inhibition of epithelial restitution.

In addition, polyamines are also necessary for expression of intercellular junctions, especially E-cadherin and occludin, in intestinal epithelial cells. Polyamines regulate expression of the *E-cadherin* gene at transcription level through a process involving Ca^{2+} and c-Myc transcription factor. Polyamines regulate occludin expression by modulating the synthesis and stability of occludin protein. Increased polyamines enhance expression of intercellular junction proteins and promote function of intestinal epithelial barrier, while depletion of cellular polyamines inhibits expression of various

intercellular junction proteins and causes dysfunction of epithelial barrier, thus resulting in an increase in paracellular permeability.

References

1. Tabor, C. W. and Tabor, H. (1984) Polyamines. *Annu. Rev. Biochem.* **53,** 749–790.
2. McCormack, S. A. and Johnson, L. R. (1991) Role of polyamines in gastrointestinal mucosal growth. *Am. J. Physiol. Gastrointest. Liver Physiol.* **260,** G795–G806.
3. Wang, J. Y. and Johnson, L. R. (1990) Luminal polyamines stimulate repair of gastric mucosal stress ulcers. *Am. J. Physiol. Gastrointest. Liver Physiol.* **259,** G584–G592.
4. Li, L., Rao, J. N., Guo, X., et al. (2001) Polyamine depletion stabilizes p53 resulting in inhibition of normal intestinal epithelial cell proliferation. *Am. J. Physiol. Cell. Physiol.* **281,** C941–C953.
5. Wang, J. Y., Wang, J., Golovina, V. A., Li, L., Platoshyn, O., and Yuan, J. X. (2000) Role of K^+ channel expression in polyamine-dependent intestinal epithelial cell migration. *Am. J. Physiol. Cell. Physiol.* **278,** C303–C314.
6. Rao, J. N., Li, L., Golovina, V. A., et al. (2001) Ca^{2+}-RhoA signaling pathway required for polyamine-dependent intestinal epithelial cell migration. *Am. J. Physiol. Cell. Physiol.* **280,** C993–C1007.
7. Fleischmann, B. K., Washabau, R. J., and Kotlikoff, M. I. (1993) Control of resting membrane potential by delayed rectifier potassium currents in ferret airway smooth muscle cells. *J. Physiol.* **469,** 625–638.
8. Freedman, B. D., Fleischmann, B. K., Punt, J. A., Gaulton, G., Hashimoto, Y., and Kotlikoff, M. I. (1995) Identification of Kv1.1 expression by murine CD4-CD8- thymocytes: a role for voltage-dependent K^+ channels in murine thymocyte development. *J. Biol. Chem.* **270,** 22406–22411.
9. Isom, L. L., DeJongh, K. S., and Catterall, W. A. (1994) Auxiliary subunits of voltage-gated ion channels. *Neuron* **12,** 1183–1194.
10. Sansom, M. S. P., Shrivastava, I. H., Bright, J. N., Tate, J., Capener, C. E., and Biggin, P. C. (2002) Potassium channels: structures, models, simulations. *Biochim. Biophys. Acta* **1565,** 294–307.
11. Rao, J. N., Platoshyn, O., Li, L., et al. (2002) Activation of K^+ channels and increased migration of differentiated intestinal epithelial cells after wounding. *Am. J. Physiol. Cell. Physiol.* **282,** C885–C898.
12. Brown, E. M., Vassilev, P. M., and Hebert, S. C. (1995) Calcium as extracellular messengers. *Cell* **83,** 679–682.
13. Bootman, M. D. and Berridge, M. J. (1995) The elemental principles of calcium signaling. *Cell* **83,** 675–678.
14. Parekh, A. B. and Penner, R. (1997) Store depletion and calcium influx. *Physiol. Rev.* **77,** 901–930.
15. Rao, J. N. and Wang, J. Y. (2002) Ca^{2+} signaling in epithelial restitution. In: *Frontiers of Gastrointestinal Research Gastrointestinal Mucosal Repair and Experimental Therapeutics* (Cho, C. H., and Wang, J. Y., eds.), S. Karger AG, Basel, Switzerland, pp. 29–42.
16. Rao, J. N., Platoshyn, O., Golovina, V. A., et al. (2006) TRPC1 functions as a store-operated Ca^{2+} channel in intestinal epithelial cells and regulates early mucosal restitution after wounding. *Am. J. Physiol. Gastrointest. Liver Physiol.* **290,** 6779–6789.
17. Zhu, X., Jiang, M., Peyton, M., et al. (1996) Trp, a novel mammalian gene family essential for agonist-activated capacitative Ca^{2+} entry. *Cell* **85,** 661–671.

18. Birnbaumer, L., Zhu, X., Jiang, M., et al. (1996) On the molecular basis and regulation of cellular capacitative Ca^{2+} entry: Roles for Trp proteins. *Proc. Natl. Acad. Sci. USA* 1**93,** 15,195–15,202.
19. Okada, T., Inoue, R., Yamazaki, K., et al. (1999) Molecular and functional characterization of a novel mouse transient receptor potential protein homologue TRP7. *J. Biol. Chem.* **274,** 27,359–27,370.
20. Rao, J. N., Li, L., Li, J., Bass, B. L., and Wang, J. Y. (1999) Differentiated intestinal epithelial cells exhibit increased migration through polyamines and myosin II. *Am. J. Physiol. Gastrointest. Liver Physiol.* **277,** G1149–G1158.
21. Johnson, L. R. (1988) Regulation of gastrointestinal mucosal growth. *Physiol. Rev.* **68,** 456–502.
22. Wang, J. Y. (2002) Expression of early primary response genes in healing of gastrointestinal mucosal injury. In: *Gastrointestinal Mucosal Repair and Experimental Therapeutics* (Cho, C. H., and Wang, J. Y., eds.), S. Karger AG, Basel, Switzerland, pp. 82–100.
23. Kemler, R. (1993) From cadherins to catenins: Cytoplasmic protein interactions and regulation of cell adhesion. *Trends Genet.* **9,** 317–321.
24. Guo, X., Rao, J. N., Liu, L., et al. (2002) Polyamines regulate β-catenin tyrosine phosphorylation via Ca^{2+} during intestinal epithelial cell migration. *Am. J. Physiol. Cell. Physiol.* **283,** C722–C734.
25. Guo, X., Rao, J. N., Liu, L., et al. (2003) Regulation of adherens junctions and epithelial paracellular permeability: a novel function for polyamines. *Am. J. Physiol. Cell. Physiol.* **285,** C1174–C1187.
26. Guo, X., Rao, J. N., Liu, L., et al. (2005) Polyamines are necessary for synthesis and stability of occludin protein in intestinal epithelial cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* **288,** G1159–G1169.
27. Alexander, J. S., Blaschuk, O. W., and Haselton, F. R. (1993) An N-cadherin-like protein contributes to solute barrier maintenance in cultured endothelium. *J. Cell. Physiol.* **156,** 610–618.
28. Man, Y., Hart, V. J., Ring, C. J. A., Sanjar, S., and West, M. R. (2000) Loss of epithelial integrity resulting from E-cadherin dysfunction predisposes airway epithelial cells to adenoviral infection. *Am. J. Respir. Cell. Mol. Biol.* **23,** 610–617.
29. Nelson, W. J. (1994) Regulation of cell adhesion and development of epithelial cell surface polarity. *Curr. Top. Membr.* **41,** 123–142.
30. Gumbiner, B. M., Stevenson, B., and Grimaldi, A. (1988) The role of cell adhesion molecule uvomorulin in the formation and maintenance of the epithelial junctional complex. *J. Cell. Biol.* **107,** 1575–1587.
31. Takeichi, M. (1990) Cadherins: A molecular family important in selective cell-cell adhesion. *Annu. Rev. Biochem.* **59,** 237–252.
32. Rothen-Rutishauser, B., Riesen, F. K., Braun, A., Gunthert, M., and Wunderli-Allenspach, H. (2002) Dynamics of tight and adherens junctions under EGTA treatment. *J. Membr. Biol.* **188,** 151–162.
33. Sakakibara, A., Furuse, M., Saitou, M., Ando-Akatsuka, Y., and Tsukita, S. (1997) Possible involvement of phosphorylation of occludin in tight junction formation. *J. Cell. Biol.* **137,** 1393–1401.
34. Bamforth, S. D., Kniesel, U., Wolburg, H., Engelhardt, B., and Risau, W. (1999) A dominant mutant of occludin disrupts tight junction structure and function. *J. Cell. Sci.* **112,** 1879–1888.

35. Rao, R. K., Basuroy, S., Rao, V. U., Karnaky, K. J., Jr., and Gupta, A. (2002) Tyrosine phosphorylation and dissociation of occludin-ZO-1 and E-cadherin-beta-catenin complexes from the cytoskeleton by oxidative stress. *Biochem. J.* **368,** 471–481.
36. Liu ,Y., Nusrat, A., Schnell, F. J., et al. (2000) Human junction adhesion molecule regulates tight junction resealing in epithelia. *J. Cell. Sci.* **113,** 2363–2374.
37. Balda, M. S., Flores-Maldonado, C., Cereijido, M., and Matter, K. (2000) Multiple domains of occludin are involved in the regulation of paracellular permeability. *J. Cell. Biochem.* **78,** 85–96.
38. Li, L., Liu, L., Rao, J. N., et al. (2002) JunD stabilization results in inhibition of normal intestinal epithelial cell growth through p21 after polyamine depletion. *Gastroenterol.* **123,** 764–779.
39. Wang, J. Y., McCormack, S. A., Viar, M. J., et al. (1993) Decreased expression of proto-oncogenes c-fos, c-myc, and c-jun following polyamine depletion in IEC-6 cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* **265,** G331–G338.
40. Guo, X., Liu, L., Rao, J. N., et al. (2004) Polyamines induce expression of the E-cadherin gene via c-Myc and enhance epithelial barrier function. *Gastroenterol.* **126,** A35.
41. Patel, A. R. and Wang, J. Y. (1997) Polyamines modulate transcription but not posttranscription of c-myc and c-jun in IEC-6 cells. *Am. J. Physiol. Cell. Physiol.* **273,** C1020–C1029.
42. Li, L., Rao, J. N., Bass, B. L., and Wang, J.-Y. (2001) NF-κB activation and susceptibility to apoptosis following polyamine depletion in intestinal epithelial cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* **280,** G992–G1004.
43. Batsche, E., Muchardt, C., Behrens, J., Hurst, H. C., and Cremisi, C. (1998) RB and c-Myc activate expression of the E-cadherin gene in epithelial cells through interaction with transcription factor AP-2. *Mol. Cell Biol.* **18,** 3647–3658.

22

Polyamine Block of Kir Channels

Toward a Molecular Picture

Harley T. Kurata and Colin G. Nichols

1. Inward-Rectifier Potassium Channels

Potassium channels are transmembrane proteins that selectively allow K^+ ions to pass from one side of the membrane to the other. Most K^+ channels exhibit a property termed rectification, which as a general physical term refers to a voltage dependence of conductance or resistance. In the context of biophysical studies of ion channels, this term is used to describe a voltage dependence of macroscopic currents in which currents through an ion channel flow preferentially into the cell (inward rectification) or out of the cell (outward rectification).

Many genes encoding K^+ selective channels that exhibit inward rectification have now been identified, and these are termed inward rectifiers or Kir channels *(1)*. The property of inward rectification differs significantly from the outward rectification common in the widely studied family of voltage-gated channels, which arises from voltage-dependent conformational changes ("gating") of the channel *(2)*. In contrast, it is now well accepted that the fundamental mechanism underlying inward rectification of Kir channels is blockade of the permeation pathway by intracellular Mg^{2+} and, most importantly, polyamines *(3–5)*. At depolarized membrane voltages, polyamines occlude the permeation pathway from the intracellular side of the channel and prevent permeation of K^+ ions. However, at hyperpolarized voltages, blocking polyamines are dislodged from the channel pore, allowing K^+ currents to flow.

Among the various Kir channels, there is a spectrum of sensitivity to blockade by intracellular polyamines, reflected in the degree of rectification that they exhibit, and underlying the functional role of each channel type. In some channel types, rectification is sufficiently strong that under physiological conditions, very little current flows through them at potentials positive to about –40 mV *(6)*. This classical, "strong" inward rectification has been best characterized in channels from the Kir2 subfamily, and is prominent in skeletal and cardiac myocytes and in glial cells and neurons in the central

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nervous system. Strong inward rectifiers allow cardiac muscle cells in particular to maintain a stable resting potential, whereas the greatly reduced conductance on depolarization avoids short-circuiting action potentials. Much weaker rectification is observed in Kir1, Kir6, and Kir7 subfamilies, and this reflects a weaker sensitivity to intracellular polyamines. A well-studied example of a "weak" inward rectifier is the adenosine triphosphate (ATP)-sensitive K^+ (K_{ATP}) channel, whose pore-forming subunits consist of the Kir6 channel subfamily *(7)*. K_{ATP} channels are present in all muscle cell types, in the brain, and play a central role in the regulation of insulin secretion from pancreatic β-cells *(8,9)*. In contrast to classical strong inward rectifiers, K_{ATP} channels allow significant outward K^+ currents to flow at positive potentials, even in the presence of supraphysiological concentrations of polyamines. Between these extremes, certain Kir channels exhibit intermediate rectification properties, such as the Kir3 subfamily, which is also gated by Gβγ subunits.

Years of physiological experiments characterizing the rectification properties of many Kir channels have been instrumental in developing an understanding of the physical principles underlying inward rectification. Furthermore, recent advances in understanding the three-dimensional structures of K^+ channels have provided new tools in understanding inward rectification, but also new constraints on models to explain this process. In this chapter, we review the identification of basic structural determinants of inward rectification, and extend these principles in the context of recently determined three-dimensional structures of inwardly rectifying K^+ channels.

2. Intracellular Polyamines Underlie Physiological Inward Rectification

Although cloning of a voltage-gated potassium channel (*Shaker*) was first reported in 1988, it was not until 1993 that the first inwardly rectifying K^+ channels were successfully cloned *(10,11)*. This advance allowed high-level expression and characterization of channels in heterologous expression systems and led rapidly to the demonstration that polyamines are the cause of inward rectification. In studies of both heterologously expressed and native strong inwardly rectifying K^+ channels, it was observed that inward rectification gradually disappeared after excision of a membrane patch from the cell *(4,12)*. A further critical observation was that placing excised membrane patches close to the surface of an oocyte or other cells could restore rectification *(4)*. These data suggested that, rather that being an intrinsic gating mechanism (as is the case for voltage dependence of outward rectification of Kv channels), inward rectification was caused by one or more soluble factors.

Preliminary biochemical purification indicated that these factors were small organic amines, and application of naturally occurring polyamines (spermine, putrescine, cadaverine) to the intracellular face of excised "inside-out" membrane patches containing Kir2 channels was demonstrated to be sufficient to restore all the essential features of inward rectification *(4–6,13)*. Steeply voltage-dependent rectification of Kir2 channels can be induced by application of micromolar spermine and spermidine to the cytoplasmic face of excised membrane patches. Although less effective and less steeply voltage-dependent, putrescine and cadaverine also cause inward rectification *(4,14)*. Both the voltage dependence and time course of spermine and spermidine unblock

match the relaxation time constants of inward rectifiers observed in intact cells, providing further evidence that polyamine block causes inward rectification in vivo *(4,5)*. In contrast to the classical strong inward rectifiers, weakly rectifying channels, such as Kir1.1 (ROMK1) and K_{ATP} channels, all exhibit very weak affinity for spermine and other polyamines, and a much shallower voltage dependence of polyamine block. Polyamines have also been demonstrated to cause inward rectification in AMPA/kainate receptors and cyclic nucleotide-gated channels, although block of these channels is not as potent as the block of Kir channels *(15,16)*.

Experimental manipulation of polyamine levels in cells provides further evidence that endogenous polyamines underlie inward rectification. For example, inhibition of the polyamine synthetic enzyme *S*-adenosylmethionine decarboxylase leads to an increase in cellular putrescine and decrease in spermidine and spermine levels, and consistent with polyamines being a critical element for inward rectification, Bianchi et al. demonstrated that this treatment resulted in relief of inward rectification of endogenous currents in RBL-1 cells *(17)*. Shyng et al. reported similar findings, using a Chinese hamster ovary cell line that is deficient in ornithine decarboxylase activity *(18)*. This cell line requires putrescine in the medium for normal cell growth; removal of putrescine leads to a gradual decline in intracellular levels of putrescine, then spermidine, and finally spermine. Experimental depletion of polyamines correlated with alterations in Kir2.3 kinetics predicted from excised-patch experiments *(18)*. Finally, manipulation of polyamine levels by genetic approaches in mouse models has provided the most recent evidence of a critical role for polyamines in the regulation of inward rectification in intact tissues. As an example, the Gyro mutant mouse strain lacks the spermine synthase gene, produces no endogenous spermine, and exhibits a significant reduction in the voltage dependence of inward rectification *(19)*.

3. Structural Features of Inward-Rectifier K^+ Channels

All K^+ channels are tetrameric arrangements of a core pore-forming domain, consisting of two transmembrane helices, a "pore loop," and cytoplasmic N- and C-termini *(20)*. Voltage-gated K^+ (Kv) channels contain additional transmembrane helices with charged residues that sense membrane voltage, but Kir channel subunits contain only the core domain. The two transmembrane helices of the core domain (M1 and M2, corresponding to the pore-forming S5 and S6 segments of Kv channels) line the pathway of ion permeation through the membrane *(2)*. Recent determination of atomic resolution structures for Kir3.1 (cytoplasmic domains only) and the bacterial KirBac1.1 (cytoplasmic and transmembrane domains; Fig. 1A) inward-rectifier K^+ channels has illuminated many unique and interesting properties of the Kir channel pore *(21,22)*. Kir channels exhibit the characteristic re-entrant pore loop structure that forms the selectivity filter of the channel. The selectivity filter underlies the ability of the channel to selectively permeate K^+ in the presence of other physiological cations, such as Na^+ *(23)*. Atomic resolution structures of Kir3.1 and KirBac1.1 suggest that the cytoplasmic N- and C-termini of Kir channels line a wide pore that extends the ion permeation pathway well beyond the width of the plasma membrane (Fig. 1A), and also form binding sites for multiple physiological channel ligands including ATP, Gβγ subunits, and anionic phospholipids such as PIP2.

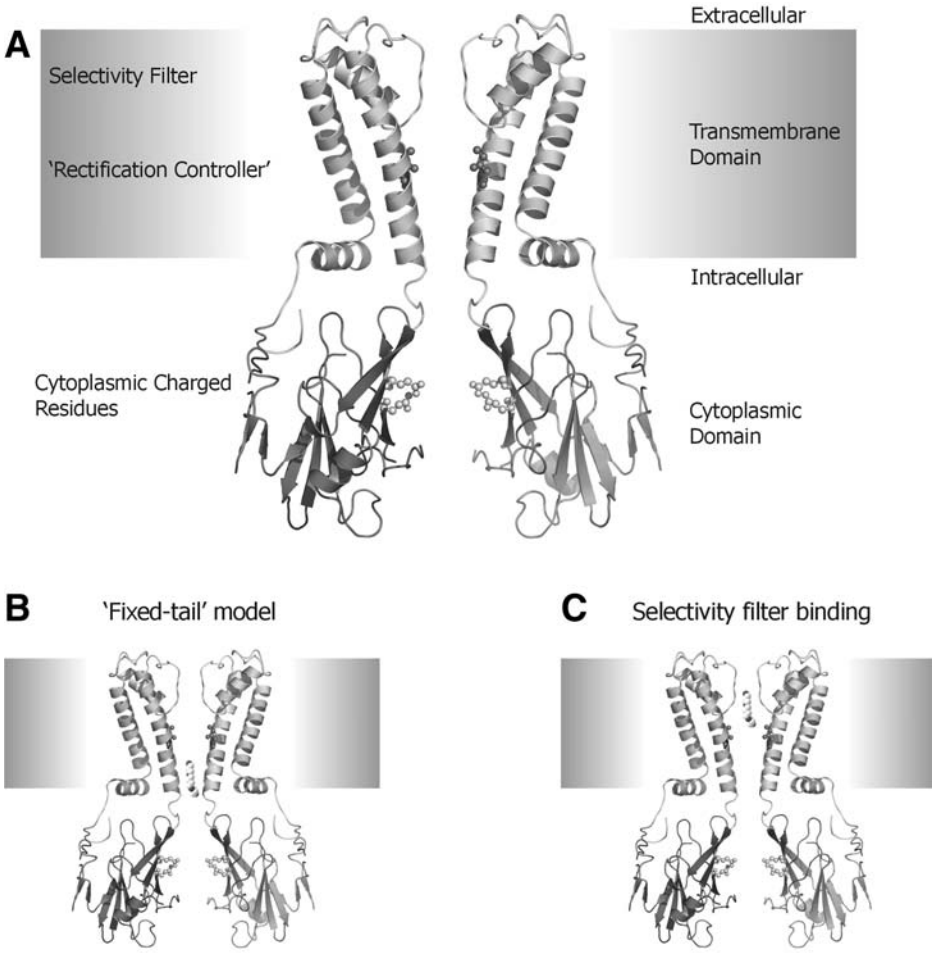

Fig. 1. Kir channel structure. **(A)** Ribbon diagram of two opposing KirBac1.1 subunits (PDB: 1P7B) *(21)*. The channel is divided into two distinct domains, the transmembrane domain formed by the M1 and M2 helices, and the cytoplasmic domain formed by the N- and C-termini. Conserved locations of pore lining negatively charged residues (the rectification controller and cytoplasmic charged residues) are illustrated in ball-and-stick format. **(B**,**C)** Potential locations of polyamine block in the channel pore. **(B)** Fixed-tail model of polyamine block, in which a polyamine is stabilized by interactions with charged residues in the inner cavity and cytoplasmic domain. **(C)** Fixed-head model of selectivity filter binding of polyamines, in which a polyamine occupies a deep site between the selectivity filter and charged residues in the inner cavity.

Although it is now well understood that blockade by intracellular polyamines is the underlying cause of inward rectification, the determination of Kir structures at atomic resolution has led several groups to revisit the topic of polyamine blockade, with a particular focus on the detailed structural basis for this process. For the remainder of this

chapter, we discuss recent work aimed at identifying the structural determinants for high-affinity polyamine block and the detailed aspects of polyamine binding within the long Kir channel pore.

Structure–function studies have demonstrated that in some Kir channels, a negative charge present at one particular location in the pore-lining M2 helix (D172 in Kir2.1) ensures very strong rectification. This residue has been termed the rectification controller (Fig. 1A), and neutralization of this residue weakens, but may not abolish strong rectification *(1)*. In other channels, the introduction of a negative charge at the equivalent residue or nearby residues can confer properties of strong rectification to otherwise weakly rectifying channels. Examples of this effect include the N171D mutation in Kir1.1 and the N160D or N160E mutations in Kir6.2 *(24–26)*. Although wild-type Kir1.1 or Kir6.2 channels exhibit very low sensitivity to polyamine blockade, the introduction of negative charges in the inner cavity of these channels results in high-affinity polyamine blockade. Furthermore, Lu and MacKinnon have shown that when this residue is replaced with a positively charged lysine, channels exhibit a shallow intrinsic rectification but, more importantly, are polyamine insensitive *(26)*. Based on sequence homology with crystallized K^+ channels, such as KcsA or KirBac, it is now accepted that this residue lines the pore with its side chain directed toward the central axis *(21)*. In the tetrameric channel assembly, these residues thus form a negatively charged ring within the inner cavity that appears to be critical for high-affinity polyamine binding.

Although the location of the rectification controller residue is generally conserved among strongly rectifying channels, there is actually not a strict structural requirement for the location of the negative charge in the inner cavity to confer strong rectification. Using Kir6.2 as a background channel, Kurata et al. demonstrated that introduction of negatively charged glutamate residues throughout the inner cavity of the channel can confer high sensitivity to polyamine blockade *(27)*. It appears that the only requirements to confer high polyamine sensitivity are that the introduced negative charge resides within the inner cavity of the channel, and that the side chain faces toward the channel pore.

Other studies have identified negatively charged residues beyond M2, within the cytoplasmic domains of the channel (Fig. 1A), which also affect sensitivity to blockade by polyamines *(28,29)*. These residues are E224 and E299 in Kir2.1, and neutralization mutations of either or both residues can significantly reduce Mg^{2+} and polyamine sensitivity. In addition, neutralization mutations of these cytoplasmic residues can alter the permeation properties of the channel, such that they exhibit weak inward rectification even in the absence of polyamines *(30)*. Inspection of structures of the cytoplasmic domains of Kir channels shows that the tertiary protein structure brings these two residues into close proximity, and that the negatively charged side chains are likely directed toward the cytoplasmic pore of the channel *(21,22)*. In this way, these residues form a second “ring” of charge within the Kir pore, in addition to the ring of charge at the position of the rectification controller. Importantly, most Kir channel types (whether strongly or weakly rectifying) have one or more negatively charged residues at positions equivalent to Kir2.1 residues E224 and E299. Therefore, the presence of negatively

charged residues in the cytoplasmic domain is not sufficient to confer properties of strong rectification.

4. Models of Polyamine Block of Inward-Rectifier K^+ Channels

The structural arrangement of the Kir channel pore, with two rings of negative charge directed toward the central axis of the channel (Fig. 1A), has led to the proposal of several potential models to explain the structural basis of polyamine blockade. One interpretation of the experimental data presented thus far is that negative charges in both the inner cavity and cytoplasmic domain contribute to a single binding site for polyamines, with each end of a blocking polyamine stabilized by one charged ring. In such a model, the "head" amine of the blocking molecule will lie near the charged ring in the inner cavity, with the "tail" amine stabilized by the pore-lining, negatively charged residues in the cytoplasmic pore (Fig. 1B). An alternative model is that polyamine blockade requires entry of the polyamine molecule into a much deeper site in the pore, near or within the selectivity filter. In this model, the leading amine of the blocker could be stabilized within a K^+ binding site at the selectivity filter, with the trailing amine stabilized by the ring of negative charge in the inner cavity (Fig. 1C). Evidence for these two contrasting models of polyamine block are discussed in Subheadings 4.1 and 4.2.

4.1. Fixed-Tail Model of Polyamine Block

Using a thermodynamic mutant cycle analysis, Guo and colleagues *(14)* examined the energetic coupling between residue D172 in the Kir2.1 inner cavity and a series of linear (1,n) diamines. They demonstrated energetic coupling that correlated with the length of the alkyl chain of the blocking diamine, with maximal coupling observed for DA9 (1,9-diaminononane. We have employed a shorthand notation of the form "DAn" for the 1,n-diaminoalkane compounds, with DA indicating a diamine, and n indicating the length of the alkyl chain). In a subsequent study, Guo and Lu *(30)* applied the same technique to characterize the energetic coupling between linear (1,n) diamines or various naturally occurring polyamines (spermine, spermidine, and cadaverine) and the charged residues in the Kir2.1 cytoplasmic pore (E224, E299). These experiments demonstrated little or no dependence of energetic coupling at E224 and E299 on alkyl chain length. The authors interpreted these data to indicate that one (tail) amine resides at a fixed location where it interacts with E224 and E299 in the cytoplasmic vestibule, explaining the absence of any length dependence of the interaction between E224/E299 and linear diamines. It was suggested that as the alkyl chain is extended, the head amine first approaches, and then extends beyond D172 in the inner cavity, with the structure of DA9 being optimal for interaction with D172. Guo and Lu *(30)* also reported a lack of any length dependence of energetic coupling of monoamines, and explained this by assuming that the single amine resides also at a fixed location in the cytoplasmic pore, and that the alkyl tail that protrudes into the inner cavity. Guo et al. *(21)* first proposed that the tail amine bound in close proximity to the ring of charge corresponding to residues E224/E299, However, after determination of the atomic resolution structure of KirBac1.1, it became apparent that the distance between the

charged rings within the Kir pore was likely approx 40Å (Fig. 1A). This is more than twice the length of a fully extended spermine molecule, and considerably longer than DA9. In light of this inconsistency between the size of even the longest physiological polyamine, and the likely distances between the two rings of charge in the pore, structural interpretations of energetic coupling experiments in the context of the fixed tail model remain unclear.

4.2. Fixed-Head Model of Polyamine Block

An alternative structural model of polyamine blockade is that polyamines bind to a very deep site in the channel pore, either near or within the selectivity filter. In this case, the head amine would be fixed by binding at the top of the inner cavity, and the trailing amine would extend back towards the inner cavity (Fig. 1C). The two models—fixed tail amine binding at the cytoplasmic ring or polyamine binding at the top of the inner cavity—make completely opposite predictions regarding alkyl chain length dependence of energetic coupling to the rectification controller residue in the inner cavity. A simple prediction of the first model is that moving the negative charge in the inner cavity towards the selectivity filter of the channel should shift the chain length dependence of energetic coupling to longer diamines; moving the location of the rectification controller to a more shallow site in the pore (toward the intracellular entrance of the channel) should shift the length dependence of energetic coupling to shorter diamines. The opposite would be predicted for the second model. As mentioned, the introduction of negative charges at pore-lining positions throughout the inner cavity and up to the entrance of the selectivity filter is sufficient to introduce strong polyamine-mediated rectification in Kir6.2 channels, allowing for a direct test of these alternatives *(27)*. Importantly, these experiments did not reveal a peak of energetic coupling at any single diamine length, in contrast to the previous work in Kir2.1 *(27,30)*. However, when negatively charged residues were introduced at either end of the Kir6.2 inner cavity (e.g., residue 129 at the intracellular entrance to the selectivity filter; residue 168 at the entrance of the inner cavity), there were clear trends in the diamine length dependence of energetic coupling that were exactly opposite to those predicted by the fixed tail hypothesis. Rather, there was clearly stronger energetic coupling to shorter diamines when negative charges were introduced near the selectivity filter. This trend was reversed, with stronger coupling to longer diamines when negative charges were introduced at more shallow sites in the inner cavity *(27)*. The detailed differences in the diamine length dependence of energetic coupling between Kir6.2 and Kir2.1 are unclear, but the more complete scan of residues in the inner pore of Kir6.2 clearly supports the alternative model in which the head amine binds at a deep site in the pore, near or possibly within the selectivity filter.

Additional experimental data and observations provide support for a model of polyamine binding to a deep site in the pore. First, this model agrees more reasonably with the dimensions of the channel as determined from X-ray crystal structures. That is, the distance between the selectivity filter and the rectification controller residue (D172 in Kir2.1) closely matches the length of an extended spermine molecule, consistent with the proposal that spermine indeed binds between the selectivity filter and

residue D172 *(31)*. Further consistency arises from the effects of positive charges in the inner cavity on strong rectification. Using sulfhydryl-reactive methanethiosulfonate reagents to modify cysteines at specific locations in a Kir pore, Kurata et al. *(27)* also examined the effects of introducing positive charges on the affinity and kinetics of spermine blockade. Introduction of positive charges at Kir6.2 residues 129 or 157, between the selectivity filter and the rectification controller, significantly reduced spermine affinity, and dramatically accelerated the kinetics of spermine block and unblock. These effects suggest that the introduction of positive charges at sites deep in the inner cavity disrupt the binding site for polyamines. They also contrast dramatically with the effects of introduction of positive charge at more shallow sites. In particular, 2-aminoethylmethanethiosulfonate modification at Kir6.2 residues 169 (at the cytoplasmic side of the inner cavity) or 212 (in the cytoplasmic pore) has no effects on spermine affinity, but significantly slows block and unblock. Overall, this is consistent with the interpretation that positive charges at these locations do not disrupt the spermine binding site, but introduce a barrier for spermine entry and exit from a binding site that is deeper in the pore.

5. Voltage Dependence of Polyamine Block: Interactions of Blocking and Permeant Cations

A coherent structural model of polyamine-induced rectification must also be able to explain the strong voltage dependence of polyamine blockade relative to other blockers, such as Mg^{2+} or quaternary ammonium compounds. This important property of rectification refers to the steepness of the conductance–voltage (g-V) relationship of strongly rectifying channels in the presence of various blocking agents. Although blockade by Mg^{2+} has a relatively shallow voltage dependence (an effective valence of 1–2), spermine blockade of strong inward rectifiers exhibits an effective valence as high as five elementary charges. Early models describing voltage-dependent blockade of ion channels involved only the movement of the blocker itself within the electric field *(32)*. In such a model, the maximum valence of block is limited to the charge of the blocking molecule, and would result from movement of the blocker through the entire transmembrane electric field. It is clear that for block by polyamines, and particularly diamines, there is a higher valence than can be accounted for by the blocker itself. As an example, DA12 carries a maximum charge of +2, but blocks Kir2.1 with an effective valence of approx 3–4 *(30,33)*. It has been proposed that this excess charge movement arises from interactions of the blocking molecule with permeant ions in the Kir pore *(33,34)*. That is, excess charge movement associated with channel block results from the associated obligate movement of permeant ions through the field, ahead of the blocking ion, as the blocking ion moves toward its binding site *(34,35)*. Coupled movement of blocking and permeant ions is reflected in the observation that the voltage dependence of inward rectification in Kir channels is modified by changes in extracellular K^+ concentration *(36)*. The demonstration that effects of external ion concentration on the kinetics of blocker dissociation are highly specific for K^+, whereas the effects of internal cations are much less species dependent, further suggests that blocker movement through the channel pore is coupled to movement of permeant ions within the selectivity filter *(35,37)*.

Based on the realization that the Kir channel pore extends at least 30 Å below the inner cavity through a cytoplasmic vestibule *(21)*, the notion of coupled movement of blockers and permeant ions has been invoked with the hypothesis that polyamine molecules may block the channel within the cytoplasmic pore *(22,30)* or at the lower end of the inner cavity, as in the fixed-tail model described previously *(14)*. In either instance, the charge movement associated with polyamine block must result entirely from the "pushing" of a column of four or five K^+ ions (which implicitly extends from the selectivity filter all the way back into the cytoplasmic pore) through the electric field, and charged elements of the blocking polyamine itself would then make little or no contribution to the overall valency. It is generally accepted that K^+ channels are multi-ion pores, such that several K^+ ions are present in single file in the pore at any given time. However, no defined K^+ binding sites have been identified below the inner cavity in any K^+ channel, and therefore it is unclear at present whether as many as four or five K^+ ions can be present simultaneously in the pore of a Kir channel. Based on studies of the bacterial K^+ channel KcsA, there are likely no more than three single file K^+ ions occupying the inner cavity and selectivity filter at any time *(23)*, which is insufficient to account for the valence of spermine blockade. Furthermore, unidirectional flux measurements (functional experiments designed to assess the number of ions residing in a multi-ion pore) result in an Ussing flux ratio exponent of 2.2 for Kir2.1 channels *(38)*, which implies that only two or three ions permeate the pore in single file. With this in mind, it has been argued that there is not a strict requirement for true single filing of K^+ ions throughout the Kir pore for a large effective valence of blockade, so long as permeant and blocking ions cannot bypass each other *(39)*. In other words, incoming polyamine ions could cause obligate movement of unordered K^+ ions from the cytoplasmic pore into the inner cavity. However, the width of the inner cavity and cytoplasmic vestibule is expected to be greater than 10 Å, based on the atomic resolution KirBac structure *(21)*, and so it is unclear whether this condition exists in an intact channel. Finally, crystallographic evidence in KcsA suggests that blocking sites for quaternary ammonium ions and divalent cations (e.g., Mg^{2+}, Ba^{2+}) are within the inner cavity (near the level of the rectification controller) and at the cytoplasmic side of the selectivity filter, respectively *(40,41)*. These blockers exhibit a valence of approximately two or less in Kir2.1 channels, and if they occupy blocking sites similar to those in KcsA, the considerably higher valence of spermine block would suggest a much deeper binding site for spermine.

Calculations based on the X-ray structures of open and closed K^+ channel pores indicate that 70–90% of the membrane field exists across the selectivity filter *(42)*. We can thus estimate charge movement expected for diamine/polyamine occupancy of K^+ binding sites in the inner cavity or selectivity filter, with the simple assumptions that the electric field in Kir channels is relatively fixed and similar to that calculated for MthK (or KcsA), and that K^+ ion occupancy is also similar in the absence of polyamine. Importantly, these calculations do not posit additional K^+ binding sites in the Kir pore, nor entry of "unordered" K^+ ions from the cytoplasmic into the inner cavity to account for excess charge movement. In particular, if the polyamine head reaches deep enough into the selectivity filter, then the combined elementary charge movement from

displacement of K^+ and entry of charged amines into the field could be as high as four, similar to voltage dependence estimated from fits of the Boltzmann equation to steady-state g–V relationships *(6,30,33,43)*. Thus movement of no more than three K^+ ions from the selectivity filter and the inner cavity ion binding site, along with the charge carried into the field by the polyamine itself, may be adequate to explain the charge movement associated with the block, without invoking obligate movement of multiple ions from the inner cavity or cytoplasmic vestibule.

Calculations of polyamine and permeant ion binding in three-dimensional models of closed and open Kir channels further suggest that polyamines experience very low energetic barriers for entry into the inner cavity and only small barriers for entry into the selectivity filter *(44,45)*. Furthermore, models of both Kir3.1 and Kir6.2[N160D] channels predict stable binding of spermine within the selectivity filter, but suggest that significant barriers within the selectivity filter normally preclude significant spermine permeation *(44,45)*. In addition, these calculations predict that longer diamines (and monoamines) are stable at progressively deeper sites in the filter, which may partially account for the increased valence of blockade observed for progressively longer diamines. In contrast, models of WT Kir6.2 channels (weak inward rectifiers) predict a large barrier for entry into the inner cavity and unstable binding of spermine in the selectivity filter and inner cavity *(44)*. Therefore, the presence of a negative charge in the inner cavity may promote spermine block by both reducing barriers for entry, and by providing some stabilization to spermine molecules that reach the selectivity filter. In entering the filter, polyamines and diamines would themselves move significant amounts of charge into the electric field, as well as discharging K^+ ions from the inner cavity and selectivity filter to the outside, leaving only externally located K^+ binding sites free. Such a model thus provides an intuitive basis for both the external $[K^+]$ dependence of block, and the excess charge movement associated with block. Although crystallographic evidence is ultimately necessary, this model of selectivity filter binding of polyamines provides a consistent explanation of the experimental features of polyamine-induced rectification of Kir channels.

6. A Role for Charged Residues in the Cytoplasmic Vestibule

Although selectivity filter binding provides an attractive model for polyamine block, it does not delineate any role for negative charges in the cytoplasmic pore. Neutralization of these residues clearly influences the kinetics and affinity of spermine blockade in Kir2.1 *(28)*, but the mechanism underlying this phenomenon has provoked considerable debate. Whether the E224/E299 residues provide actual binding sites for blocking polyamine *(14,30)* or act to facilitate entry to the deeper sites *(43,46)* is unclear. That the E224/E299 residues may provide a binding site for polyamines was discussed earlier in Subheading 4.1., in the context of the fixed tail model of polyamine block. However, Xie and colleagues have demonstrated that interactions of polyamines at sites within the cytoplasmic vestibule reduce single-channel conductance without completely blocking the channel pore and suggested that these interactions facilitate spermine entry toward a blocking site deeper in the pore *(43,46)*. Although this has been echoed elsewhere in the literature, we would rephrase this hypothesis somewhat to

suggest that peripheral interactions of polyamines in the cytoplasmic pore may provide a concentrating effect, reducing the apparent K_D for spermine at a deeper blocking site by effectively concentrating the blocker outside the inner cavity. A similar mechanism has been described in the "large-conductance" MthK and BK channels, in which rings of negative charges increase single-channel conductance and promote outward K^+ currents by concentrating K^+ at the entrance to the inner cavity *(47,48)*. Incidentally, E224 and E299 appear to be playing a similar role in Kir2.1 because neutralization of one or both reduces single-channel conductance and introduces intrinsic inward rectification *(46,49)*. Collectively, these data suggest that the cytoplasmic pore may act to concentrate both permeant and blocking ions, thereby controlling polyamine entry to and exit from deeper binding sites.

7. A Role for Charged Residues Above the Inner Cavity

Several residues have been identified that stabilize the structure of the selectivity filter and that are required for polyamine block of Kir channels. This set of observations provides further consistency with the notion that polyamine binding at or in the selectivity filter underlies strong rectification. In Kir channels, immediately before the GYG signature sequence that forms the K^+ channel selectivity filter, there is a glutamate residue that is predicted to make a salt bridge with an arginine immediately beyond the GYG sequence. In Kir2.1, mutation of the glutamate (E138) abolishes channel function *(50)*. Channel activity can be rescued by mutation of the paired arginine (i.e., the double mutant, E138R/R148E), but this double mutant loses selectivity for K^+ and intracellular polyamines no longer result in strong rectification *(50)*. Similar effects have been demonstrated in Kir3.1/3.4 channels *(45)*, where mutation of the equivalent charged residues can cause a dramatic loss of ion selectivity and of inward rectification. Furthermore, this disruption of the selectivity filter also allows the channels to carry measurable spermine currents. These residues are unlikely to make contact with polyamines or permeant ions themselves, rather it has been suggested that they serve to control the conformation of the selectivity filter *(45)*. Molecular modeling of ion permeation suggests that disruption of this salt bridge may alter the flexibility of the selectivity filter, causing loss of both K^+ ion selectivity and tight polyamine binding in the filter, such that polyamines may now experience essentially no barrier to permeation *(45)*. Because disruption of the selectivity filter results in significant spermine permeation, the clear suggestion is that the selectivity filter presents a significant terminal barrier for spermine to escape the channel pore into the extracellular medium.

8. Summary

Although it is well understood that blockade of Kir channels by intracellular polyamines underlies inward rectification, the structural details of this blockade remain controversial. Conflicting hypotheses, in which polyamines bind either within the selectivity filter or at a shallower site in the Kir pore, have been proposed to explain the high affinity and steep-voltage dependence of polyamine block in strongly rectifying channels. Our own studies show that introduction of negative charges at residues throughout the inner cavity significantly strengthen block by polyamines.

Also, introduction of positive charges at the entrance to the selectivity filter cause dramatic loss of polyamine affinity, whereas introduction of positive charges near the entrance to the inner cavity slow the kinetics of polyamine block, but do not change steady-state rectification. Based on these most recent results, we favor a model in which strong rectification results from blocking of the permeation pathway by polyamines in the inner cavity and selectivity filter. A model of polyamine binding within the selectivity filter provides a straightforward explanation for both the uniquely steep voltage dependence of polyamine block and the dependence on external $[K^+]$, the defining features of classical inward rectification.

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References

1. Nichols, C. G. and Lopatin, A. N. (1997) Inward rectifier potassium channels. *Ann. Rev. Physiol.* **59,** 171–191.
2. Yellen, G. (2002) The voltage-gated potassium channels and their relatives. *Nature* **419,** 35–42.
3. Vandenberg, C. A. (1987) Inward rectification of a potassium channel in cardiac ventricular cells depends on internal magnesium ions. *Proc. Natl. Acad. Sci. USA* **84,** 2560–2564.
4. Lopatin, A. N., Makhina, E. N., and Nichols, C. G. (1994) Potassium channel block by cytoplasmic polyamines as the mechanism of intrinsic rectification. *Nature* **372,** 366–369.
5. Fakler, B., Brandle, U., Glowatzki, E., Weidemann, S., Zenner, H. P., and Ruppersberg, J. P. (1995) Strong voltage-dependent inward rectification of inward rectifier K^+ channels is caused by intracellular spermine. *Cell* **80,** 149–154.
6. Lopatin, A. N., Makhina, E. N., and Nichols, C G. (1995) The mechanism of inward rectification of potassium channels—Long-pore plugging by cytoplasmic polyamines. *J. Gen. Physiol.* **106,** 923–955.
7. Seino, S., Iwanaga, T., Nagashima, K., and Miki, T. (2000) Diverse roles of K(ATP) channels learned from Kir6.2 genetically engineered mice. *Diabetes* **49,** 311–318.
8. Ashcroft, F. M., Harrison, D. E., and Ashcroft, S. J. (1984) Glucose induces closure of single potassium channels in isolated rat pancreatic β-cells. *Nature* **312,** 446–448.
9. Nichols, C. G. and Koster, J. C. (2002) Diabetes and insulin secretion: Whither KATP? *Am J. Physiol. Endocrinol. Metab.* **283,** E403–E412.
10. Kubo, Y., Baldwin, T. J., Jan, Y. N., and Jan, L. Y. (1993) Primary structure and functional expression of a mouse inward rectifier potassium channel. *Nature* **362,** 127–133.
11. Ho, K., Nichols, C. G., Lederer, W. J., et al. (1993) Cloning and expression of an inwardly rectifying ATP-regulated potassium channel. *Nature* **362,** 31–38.
12. Matsuda, H. (1988) Open-state substructure of inwardly rectifying potassium channels revealed by magnesium block in guinea-pig heart cells. *J. Physiol.* **397,** 237–258.
13. Ficker, E., Taglialatela, M., Wible, B. A., Henley, C. M., and Brown, A. M. (1994) Spermine and spermidine as gating molecules for inward rectifier K^+ channels. *Science* **266,** 1068–1072.

14. Guo, D. and Lu, Z. (2003) Interaction mechanisms between polyamines and IRK1 inward rectifier K^+ channels. *J. Gen. Physiol.* **122,** 485–500.
15. Bowie, D. and Mayer, M. L. (1995) Inward rectification of both AMPA and kainate subtype glutamate receptors generated by polyamine-mediated ion channel block. *Neuron* **15,** 453–462.
16. Guo, D. and Lu, Z. (2000) Mechanism of cGMP-gated channel block by intracellular polyamines. *J. Gen. Physiol.* **115,** 783–798.
17. Bianchi, L., Roy, M. L., Taglialatela, M., Lundgren, D. W., Brown, A. M., and Ficker, E. (1996) Regulation by spermine of native inward rectifier K^+ channels in RBL-1 cells. Library. *J. Biol. Chem.* **271,** 6114–6121.
18. Shyng, S. L., Sha, Q., Ferrigni, T., Lopatin, A. N., and Nichols, C. G. (1996) Depletion of intracellular polyamines relieves inward rectification of potassium channels. *Proc. Natl. Acad. Sci. USA* **93,** 12,014–12,019.
19. Lopatin, A. N., Shantz, L. M., Mackintosh, C. A., Nichols, C. G., and Pegg, A. E. (2000) Modulation of potassium channels in the hearts of transgenic and mutant mice with altered polyamine biosynthesis. *J. Mol. Cell. Cardiol.* **32,** 2007–2024.
20. Doyle, D. A., Morais Cabral, J., Pfuetzner, R. A., et al. (1998) The structure of the potassium channel: molecular basis of K^+ conduction and selectivity. *Science* **280,** 69–77.
21. Kuo, A., Gulbis, J. M., Antcliff, J. F., et al. (2003) Crystal structure of the potassium channel KirBac1.1 in the closed state. *Science* **300,** 1922–1926.
22. Nishida, M. and MacKinnon, R. (2002) Structural basis of inward rectification. Cytoplasmic pore of the G protein-gated inward rectifier GIRK1 at 1.8 Å resolution. *Cell* **111,** 957–965.
23. Zhou, Y., Morais-Cabral, J. H., Kaufman, A., and MacKinnon, R. (2001) Chemistry of ion coordination and hydration revealed by a K^+ channel- Fab complex at 2.0 Å resolution. *Nature* **414,** 43–48.
24. Shyng, S., Ferrigni, T., and Nichols, C. G. (1997) Control of rectification and gating of cloned KATP channels by the Kir6.2 subunit. *J. Gen. Physiol.* **110,** 141–153.
25. Wible, B. A., Taglialatela, M., Ficker, E., and Brown, A. M. (1994) Gating of inwardly rectifying K^+ channels localized to a single negatively charged residue. *Nature* **371,** 246–249.
26. Lu, Z. and MacKinnon, R. (1994) Electrostatic tuning of Mg^{2+} affinity in an inward-rectifier K^+ channel. *Nature* **371,** 243–246.
27. Kurata, H. T., Phillips, L. R., Rose, T., et al. (2004) Molecular basis of inward rectification: polyamine interaction sites located by combined channel and ligand mutagenesis. *J. Gen. Physiol.* **124,** 541–554.
28. Kubo, Y. and Murata, Y. (2001) Control of rectification and permeation by two distinct sites after the second transmembrane region in Kir2.1 K^+ channel. *J. Physiol.* **531,** 645–660.
29. Yang, J., Jan, Y. N., and Jan, L. Y. (1995) Control of rectification and permeation by residues in two distinct domains in an inward rectifier K^+ channel. *Neuron* **14,** 1047–1054.
30. Guo, D., Ramu, Y., Klem, A. M., and Lu, Z. (2003) Mechanism of rectification in inward-rectifier K^+ channels. *J. Gen. Physiol.* **121,** 261–276.
31. John, S. A., Xie, L. H., and Weiss, J. N. (2004) Mechanism of inward rectification in Kir channels. *J. Gen. Physiol.* **123,** 623–625.
32. Woodhull, A. M. (1973) Ionic blockage of sodium channels in nerve. *J. Gen. Physiol.* **61,** 687–708.
33. Pearson, W. L. and Nichols, C. G. (1998) Block of the Kir2.1 channel pore by alkylamine analogues of endogenous polyamines. *J. Gen. Physiol.* **112,** 351–363.

34. Spassova, M. and Lu, Z. (1998) Coupled ion movement underlies rectification in an inward-rectifier K^+ channel. *J. Gen. Physiol.* **112,** 211–221.
35. Hille, B. and Schwarz, W. (1978) Potassium channels as multi-ion single-file pores. *J. Gen. Physiol.* **72,** 409–442.
36. Lopatin, A. N. and Nichols, C. G. (1996) [K^+] dependence of polyamine-induced rectification in inward rectifier potassium channels (IRK1, Kir2.1). *J. Gen. Physiol.* **108,** 105–113.
37. Oliver, D., Hahn, H., Antz, C., Ruppersberg, J. P., and Fakler, B. (1998) Interaction of permeant and blocking ions in cloned inward-rectifier K^+ channels. *Biophys. J.* **74,** 2318–2326.
38. Stampe, P., Arreola, J., Perez-Cornejo, P., and Begenisich, T. (1998) Nonindependent K^+ movement through the pore in IRK1 potassium channels. *J. Gen. Physiol.* **112,** 475–484.
39. Lu, Z. (2004) Mechanism of rectification in inward-rectifier K^+ channels. *Annu. Rev. Physiol.* **66,** 103–129.
40. Zhou, M., Morais-Cabral, J. H., Mann, S., and MacKinnon, R. (2001) Potassium channel receptor site for the inactivation gate and quaternary amine inhibitors. *Nature* **411,** 657–661.
41. Jiang, Y. and MacKinnon, R. (2000) The barium site in a potassium channel by x-ray crystallography. *J. Gen. Physiol.* **115,** 269–272.
42. Jiang, Y., Lee, A., Chen, J., Cadene, M., Chait, B. T., and MacKinnon, R. (2002) The open pore conformation of potassium channels. *Nature* **417,** 523–526.
43. Xie, L. H., John, S. A., and Weiss, J. N. (2003) Inward rectification by polyamines in mouse Kir2.1 channels: synergy between blocking components. *J. Physiol.* **550,** 67–82.
44. Rose, T. and Nichols, C. G. (2003) Molecular modeling of Kir channel-polyamine interactions. *Biophys. J.* **84,** 80a.
45. Dibb, K. M., Rose, T., Makary, S. Y., et al. (2003) Molecular basis of ion selectivity, block and rectification of the inward rectifier Kir3.1/Kir3.4 K^+ channel. *J. Biol. Chem.* **278,** 49,537–49,548.
46. Xie, L. H., John, S. A., and Weiss, J. N. (2002) Spermine block of the strong inward rectifier potassium channel Kir2.1: dual roles of surface charge screening and pore block. *J. Gen. Physiol.* **120,** 53–66.
47. Nimigean, C. M., Chappie, J. S., and Miller, C. (2003) Electrostatic tuning of ion conductance in potassium channels. *Biochemistry* **42,** 9263–9268.
48. Brelidze, T. I., Niu, X., and Magleby, K. L. (2003) A ring of eight conserved negatively charged amino acids doubles the conductance of BK channels and prevents inward rectification. *Proc. Natl. Acad. Sci. USA* **100,** 9017–9022.
49. Reuveny, E., Jan, Y. N., and Jan, L. Y. (1996) Contributions of a negatively charged residue in the hydrophobic domain of the IRK1 inwardly rectifying K^+ channel to K^+-selective permeation. *Biophys. J.* **70,** 754–761.
50. Yang, J., Yu, M., Jan, Y. N., and Jan, L. Y. (1997) Stabilization of ion selectivity filter by pore loop ion pairs in an inwardly rectifying potassium channel. *Proc. Natl. Acad. Sci. USA* **94,** 1568–1572.

IV

Polyamine Homeostasis and Transport

23

Regulation of Ornithine Decarboxylase Expression

Lo Persson

1. Introduction

It has become increasingly obvious during the last two decades that a multitude of cellular processes are highly dependent on adequate levels of polyamines *(1–6)*. Too high concentrations of the polyamines are toxic to the cell and may induce cell death or apoptosis *(7)*. On the other hand, too low concentrations may negatively affect anabolic events, such as the synthesis of DNA, RNA, and protein, eventually giving rise to cell-growth arrest *(6)*. Thus it is important for cells to maintain their polyamine concentrations within rather narrow limits. Polyamine homeostasis is achieved by a careful balance between synthesis, degradation, and uptake of the amines *(3)*. The introduction of molecular techniques in studies concerning polyamine metabolism has revealed a complex network of regulatory mechanisms involved in the cellular control of polyamine levels.

The first and what is often considered the rate-limiting step in the biosynthesis of the polyamines is catalyzed by the enzyme ornithine decarboxylase (ODC). ODC is highly regulated at a multitude of levels, including transcription, translation, and post-translational modification *(3,8–10)*. Some of the mechanisms involved in the regulation of ODC are truly unique and resemble those found in the control of various proto-oncogenes. Mammalian ODC has a fast turnover, with a half-life as short as a few minutes *(9,10)*. The cellular level of ODC is thus rapidly changed when the rate of synthesis or degradation is changed. The turnover of the polyamines in the cell appears to be much slower. Thus it is not clear why the cells need such a rapid regulation of the enzyme. However, it should be stressed that we do not know much about the static and dynamic distribution of the polyamines between cellular compartments. It is highly likely that part of the polyamines exists as free pools, which may change more rapidly than bound polyamines *(11)*. In any case, it is clear that the mechanisms involved in the regulation of ODC are extensive and unique in many parts, indicating the importance of this enzyme.

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2. Transcriptional Regulation

ODC is induced by a large variety of treatments and agents affecting cell growth or metabolism. The induction is usually, at least partly, caused by an increased transcription of the *ODC* gene resulting in increased levels of ODC messenger RNA (mRNA) *(12)*, although a stabilization of the ODC mRNA has also been shown to contribute to the rise in ODC mRNA content in some experimental systems *(13,14)*. The promoter region of the *ODC* gene contains responsive elements for a number of factors affecting transcription *(15)*. As with many other eukaryotic genes, the *ODC* gene contains several GC-rich regions that are likely binding sites for the transcription factor Sp1 *(15)*. The ODC promoter do also contain binding motifs for the members of the cyclic adenosine 5′-monophosphate response element binding protein/activated transcription factor family of transcription factors *(16)*. There are three putative cyclic adenosine 5′-monophosphate response elements in the mammalian *ODC* gene. Several studies have demonstrated that a variety of treatments affecting intracellular cyclic adenosine 5′-monophosphate levels, and thus the protein kinase A signaling pathway, also affect the expression of ODC mRNA *(15)*. Phorbol esters, like the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA), strongly and rapidly induce ODC activity and ODC mRNA, indicating the existence of TPA-responsive elements in the *ODC* gene *(17,18)*. Overexpression of the *ODC* gene has been suggested to result in enhanced susceptibility to skin tumorigenesis in mice *(19)*. Constitutive overexpression of ODC in the skin of transgenic mice has been demonstrated to cause increased frequency of spontaneous tumor development in the skin *(20)*. In a recent study, Guo et al. *(21)* demonstrate that mice, in which one copy of the murine *ODC* gene has been inactivated by a homologous recombination, exhibit reduced induction of epidermal ODC activity after treatment with TPA. Furthermore, when exposed to a two-stage tumor initiation–promotion protocol (7,12-dimethylbenz(a)anthracene/TPA), substantially fewer skin papillomas developed in these mice, compared with wild-type littermates. Overexpression of human ODC in NIH3T3 cells induced transformation of the cells, which was blocked by the ODC inhibitor difluoromethylornithine (DFMO) or antisense ODC RNA *(22)*. In addition, the ODC-overproducing NIH3T3 cells were tumorigenic in nude mice, giving rise to rapidly growing, large fibrosarcomas at the site of inoculation *(23)*. Thus, a clear correlation between ODC expression and tumorigenesis appears to exist, indicating that the gene encoding ODC may be considered as a proto-oncogene important for regulation of cell proliferation and transformation.

Increased ODC expression is a common phenomenon in tumor cell lines, indicating that various oncogenes may be involved in the regulation of the *ODC* gene expression. Of particular interest is c-Myc, which is overexpressed in most cancers and capable of inducing growth factor-independent cell-cycle progression and apoptotic cell death *(24)*. c-Myc together with Max forms a heterodimer that binds to the E-box sequence (CACGTG) *(25,26)*. The Myc–Max heterodimer can transactivate a number of genes containing the CACGTG motif in their promoters. The mammalian *ODC* gene has been shown to contain two or three CACGTG E-boxes and both the endogenous *ODC* gene and heterologous ODC promoter constructs are transactivated when cells are transfected with c-Myc expression vectors *(27–29)*.

Interestingly, the c-Myc-responsive CACGTG sequence in the *ODC* gene, as with that of other genes transactivated by c-Myc, is downstream of the transcription start site, indicating that downstream sequences are preferred c-Myc targets. However, a detailed study of the effects of the position showed that the degree of transactivation was not markedly influenced by the position of the responsive elements *(30)*. Instead, the extent of transactivation was dependent on the number of c-Myc-binding sites. Nilsson et al. *(31)* recently reported that in quiescent cells the c-Myc-responsive E-boxes are occupied by Max and Mnt, which function as a putative Myc antagonist. In proliferating cells this complex is displaced by Myc–Max complexes. Knockdown of Mnt expression induces accelerated proliferation and is sufficient to transform primary fibroblasts in conjunction with Ras. Thus, Mnt may act as a tumor suppressor which effects are counteracted by Myc.

c-Myc plays an important role in the regulation of cell-cycle progression as well as of apoptosis *(24,32,33)*. It is conceivable that part of c-Myc's effects are mediated through other proteins, such as ODC, which are transactivated by c-Myc *(27–29)*. It is a well-known fact that ODC is essential for cell proliferation, and increased expression of ODC has in some instances been shown to induce transformation of cells *(22)*. Thus it is possible that ODC is mediating some of c-Myc's effects on cell proliferation. Also, apoptosis induced by c-Myc appears to be, at least partly, mediated by ODC *(33–35)*. Interleukin-3 (IL-3) induces both c-Myc and ODC in 32D.3 murine myeloid cells. Withdrawal of IL-3 results in a rapid decrease in c-Myc and ODC expression, eventually followed by apoptotic cell death. Enforced expression of c-Myc during IL-3 withdrawal results in rapid apoptosis in addition to high ODC expression. In fact, enforced expression of ODC is sufficient to induce cell death after IL-3 withdrawal. This effect is inhibited by the ODC inhibitor DFMO, strongly indicating that ODC is also an important mediator of c-Myc-induced apoptosis.

Recent studies show that c-Myc and ODC expression may be involved in intestinal tumorigenesis. Most sporadic colorectal adenomas, which may develop into tumors, have acquired mutations in the adenomatous polyposis coli gene (*APC*) *(36)*. Such mutations are the cause of familial adenomatous polyposis, a hereditary form of colon cancer in humans *(37,38)*. *APC* affects the transcription of a number of genes, including c-Myc *(39)*. Wild-type *APC* suppresses c-Myc expression and stimulates the expression of the c-Myc antagonist Mad1, which forms a heterodimer with Max and as such represses the transcriptional activation by Myc *(40)*. Thus mutations in the *APC* gene (resulting in a reduced expression of the gene product) are associated with an increase activation of c-Myc target genes, including *ODC (41)*. ODC expression has been demonstrated to be increased in the mucosa of individuals with familial adenomatous polyposis, compared with normal controls *(42)*. Furthermore, Gerner and colleagues *(41)* have shown that ODC expression is increased in the intestinal mucosa of mice expressing a mutant *APC* gene, compared with normal littermates. These mice also exhibited increased intestinal tumorigenesis, which could be suppressed by treatment with the ODC inhibitor DFMO, suggesting a strong correlation between APC-dependent intestinal tumorigenesis and ODC expression *(41)*.

The human *ODC* gene contains two closely spaced c-Myc-responsive CACGTG E-boxes in the first intron *(43)*. There are two known alleles of the human *ODC* gene,

defined by a PstI restriction fragment length polymorphism polymerase chain reactions in intron 1 *(44)*. The polymorphic site is located between the two E-boxes in the intron and consists of a single A/G nucleotide polymorphism at base +316 (relative to the transcription start site) (Fig. 1). Determination of the frequencies of the A and G alleles in the US population, revealed that the majority of the individuals were homozygous (~50%) or heterozygous (~40%) for G, whereas a minority (~10%) were homozygous for A *(45)*. Funtional analysis of the two alleles, using expression of reporter gene constructs, indicated that the A allele gave a stronger response to c-Myc activation than the G allele *(44)*. In a recent study, Gerner and colleagues *(45)* demonstrated that the risk of adenoma recurrence among participants in a colon cancer prevention trial was less for individuals homozygous for the A allele, compared with those heterozygous or homozygous for the G-allele. Experimental and clinical data indicate that nonsteroidal anti-inflammatory drugs, including aspirin, may prevent or inhibit development of colorectal adenomas and tumorigenesis *(42,46,47)*. Thus aspirin may be used as a chemoprevention for colorectal cancer. In the study by Gerner and colleagues *(45)*, individuals who were taking aspirin were shown to be less likely to have an adenoma recurrence. However, when analyzed for aspirin use and for ODC genotype, a very interesting observation was made. Those homozygous for the A allele and reported using aspirin were much less likely to have adenoma recurrence (odds ratio of 0.10 compared with 1.0 and 0.83 for G homozygous nonaspirin and aspirin users, respectively). The reason for this is not known. However, it was demonstrated that the expression of wild-type *APC* in a human colon cancer-derived cell line inhibited c-Myc expression and increased the expression of Mad1, which was shown to selectively inhibit the activity of the A-variant of the ODC promoter, but not the G-variant. Thus the suppression of ODC expression may be greater in individuals homozygous for the A allele, reducing the risk for adenoma recurrence. Analysis of the effects of aspirin indicated that this drug induced the degradation of polyamines. Hence a common denominator for the effects of both aspirin and the *ODC* polymorphism on the recurrence of adenoma appeared to be the suppression of cellular polyamine levels. This is an interesting observation and forms the rationale for chemoprevention studies using DFMO alone or in combination with various nonsteroidal anti-inflammatory drugs *(48)*.

3. Translational Regulation

When ODC is induced, the total increase in ODC activity is usually much larger than that found in ODC mRNA level, suggesting also the involvement of posttranscriptional mechanisms. The increase in ODC activity is often explained by a stabilization of the enzyme against degradation. However, in some experimental systems, the rise in ODC activity cannot be explained only by an increase in ODC mRNA and a stabilization of the enzyme protein, also indicating an increase in the efficiency by which the ODC mRNA is translated *(14,49,50)*.

The polyamines exert a strong feedback control of ODC *(3,9,51)*. An excess of polyamines rapidly downregulates ODC activity, whereas a cellular polyamine deficiency induces a compensatory upregulation of ODC activity. Part of this feedback regulation of ODC is caused by changes in the turnover of the enzyme *(10)*. The

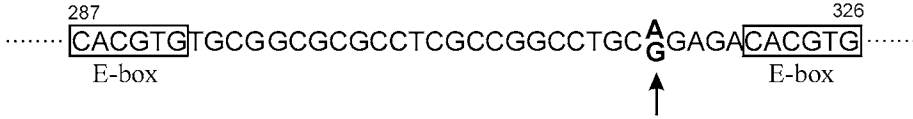

Fig. 1. Myc binding elements (E-boxes) and single A/G nucleotide polymorphism in intron 1 of the human *ODC* gene.

mechanisms involved in the polyamine-mediated control of ODC degradation have been shown to be unique to this enzyme (*see* subheading 4.). Besides changes in ODC turnover rate, the feedback control of ODC appears to involve changes in the ODC synthesis rate *(3,9,51)*. However, the polyamine-mediated changes in enzyme synthesis are not explained by any changes in the amount of ODC mRNA, suggesting that the effects of the polyamines are on the translational level rather than on the transcription or stability of the ODC mRNA *(3,51)*.

The expression of ODC is also strongly affected by the osmolarity of the growth medium *(52–54)*. Exposure of cells to a hypertonic medium rapidly results in a marked reduction of ODC activity, whereas exposure to a hypotonic growth medium results in a dramatic increase in ODC activity within a very short time after the onset of the osmotic shock. The mechanisms involved are still not fully understood. In some systems, the changes in ODC activity are correlated with changes in the ODC mRNA content, whereas in other systems the phenomenon appears to involve mainly, if not exclusively, translational and posttranslational mechanisms *(52,54)*.

Mammalian ODC mRNA, as with many of the other mRNAs encoding growth-related proteins, belongs to the rare group of mRNAs with a very long 5′ untranslated region (UTR) *(55,56)*. The 5′ UTR of mammalian ODC mRNA is about 300 nucleotides long and has a very high GC content. The high ratio of GC to AU increases the possibility of strong secondary structures being formed in this part of the mRNA. These structures may negatively affect the translation of the message. Furthermore, the 5′ UTR of ODC mRNA also contains an upstream open reading frame, which may suppress the translation. Using various expression systems, it has been shown that the 5′ UTR of ODC mRNA strongly inhibits the translation of subsequent reporter genes *(57–59)*. Most of this suppressive effect on translation is mapped to the first part of the 5′ UTR. This part of the mRNA is particularly G/C-rich and is expected to form a stable stem loop, which could negatively affect translation. Interestingly, it has been demonstrated that tissue extracts contain a 58-kDa protein that appear to bind specifically to this part of the ODC mRNA *(60)*. However, the biological function of this protein is still unknown. Most of the ODC mRNA is found associated with fractions containing ribosomal subunits and monosomes in polysome profiles, which indicates the mRNA is indeed poorly translated in vivo *(61,62)*.

The 3′ UTR of ODC mRNA is also relatively long (>300 nucleotides). However, the frequency of G and C in the 3′ UTR is much less compared with that of the 5′ UTR, which suggest that this part of the ODC mRNA contains less stable secondary structures than the 5′ UTR *(63)*. Nevertheless, it has been demonstrated that the 3′ UTR

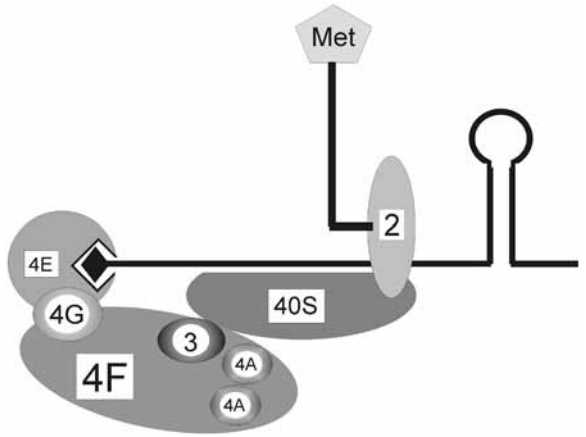

Fig. 2. The cap-dependent initiation complex. The initiation factor eIF4F consists of eIF3, eIF4A, and eIF4G.

may interact with the 5′ UTR of ODC mRNA in such a way that the suppressive effect of the 5′ UTR on translation is reduced *(57,59)*. The underlying mechanism is not yet known.

One of the limiting factors for the initiation of translation has been suggested to be the initiation factor, eIF4E *(64)*. In particular mRNAs with long and structured 5′ UTRs is affected by low levels of eIF4E *(65)*. eIF4E is important in the early process of initiation by bringing the 5′ methylated guanosine cap structure of the mRNA and the 40S ribosomal subunit closer together (Fig. 2). The eIF4E has two binding sites: one for the cap structure of the mRNA and one for another initiation factor, eIF4G, which in turn is joined to the 40S ribosomal subunit by the initiation factor eIF3. Thus the initiation factors eIF4E, eIF4G, and eIF3 form a bridge between the cap structure and the 40S ribosomal subunit. Besides binding to eIF4G, eIF3 may bind directly to the mRNA. The eukaryotic initiation factor 4G is a large protein and has binding sites for eIF4A, eIF4E, and eIF3. The initiation factor eIF4A contains a RNA helicase activity, which is important for the melting of secondary structures of the 5′ UTR. Thus the initiation factors eIF4E, eIF4G, and eIF4A form a large complex, which sometimes is referred to as eIF4F. When all the components needed for initiation are brought together the ribosomal subunit "scans" the mRNA for the correct initiation codon, starting from the cap end of the mRNA *(66)*. This scanning procedure may be suppressed by strong secondary structures in the 5′ UTR *(67)*. However, the presence of helicase activity in eIF4A facilitates a continued scanning *(68)*.

Most mRNAs coding for proto-oncogenes or other growth-related proteins have long G/C-rich 5′ UTRs. These mRNAs are especially dependent on the initiation factor eIF4E for their translation. Interestingly, a number of growth factors are known to induce the transcription of eIF4E mRNA and the promoter of the *eIF4E* gene contains c-Myc-responsive motifs, indicating that c-Myc may also transactivate this gene *(69)*. Thus eIF4E is an important factor in the control of cell growth and proliferation, which

is supported by the fact that increased expression of eIF4E stimulates DNA synthesis and cell-cycle progression, but inhibits apoptosis *(70,71)*. Furthermore, forced overexpression of eIF4E has been demonstrated to induce transformation of various rodent cell lines and thus eIF4E may play a role in tumorigenesis *(72,73)*.

As with other mRNAs coding for growth factors, the ODC mRNA has a long and highly structured 5′ UTR. Overexpression of ODC has been demonstrated to induce transformation, and may thus be considered as a proto-oncogene *(22)*. The expression of ODC has been shown to be strongly affected by eIF4E. Cells having increased levels of eIF4E exhibit increased ODC activity, which appears to be caused by reduced translational suppression exerted by the secondary structures of the ODC mRNA 5′ UTR *(74,75)*. Antisense eIF4E RNA, on the other hand, has been shown to increase the suppression of ODC mRNA translation, resulting in a decrease in cellular ODC activity *(76)*. Interestingly, the eIF4E-induced transformation appears to be related to ODC. The transformation has been demonstrated to be reversed by treatment with the ODC inhibitor DFMO or by expression of an ODC dominant–negative mutant, suggesting a role for ODC in the transformation mechanism *(77,78)*.

Most mRNAs in the cell are being translated in a cap-dependent manner. However, a minor fraction of the mRNAs appears to be translated without preceding cap binding and scanning *(79,80)*. Thus the mechanism seems to involve an "internal initiation," in which the ribosomal complex binds directly to an internal site close to the initiation codon on the 5′ UTR. This site is called an "internal ribosomal entry site" (IRES), and was first demonstrated to exist in picornavirus mRNA *(81,82)*. These mRNAs are uncapped mRNAs with a high degree of secondary structures (as well as upstream AUGs) in their 5′ UTR. Cellular infection with picornavirus results in an inhibition of the host cap-dependent translation resulting from cleavage of eIF4G by a viral protease *(82)*. However, because the IRES-dependent initiation of the picornavirus mRNA is not affected cellular protein synthesis is mainly directed toward picorna protein production. Internal initiation is not dependent on eIF4E or the amino terminal part of eIF4G (which is cleaved off by the picorna protease), but is otherwise dependent on virtually the same initiation factors as cap-dependent initiation.

An increasing number of mammalian IRES-containing mRNAs have been identified during the last decade. A large fraction of these mRNAs code for proteins involved in the regulation of cell proliferation or embryonic development (e.g., various growth factors and proto-oncogenes) *(83)*. Characteristically, their 5′ UTRs are very long and highly structured, often with one or several AUGs upstream to the main start codon. ODC mRNA also has been suggested to contain an IRES, which functions exclusively during the G2/M phase of the cell cycle *(84)*. During the cell cycle, there are two peaks of ODC activity; one during the G1/S boundary and one during the G2/M transition *(50,85)*. The first peak in ODC activity is corresponding to a general rise in protein synthesis and is probably the result of normal cap-dependent translation. However, the timing of the second peak coincides with a period of the cell cycle when the cap-dependent translation is markedly inhibited. Results from a study by Pyronnet et al. *(84)* indicated that the rise in ODC synthesis during the G2/M phase was a result of internal initiation at an IRES. This IRES was located close to the initiation AUG and demonstrated to

contain a pyrimidine-rich sequence similar to that of the picornavirus 5′ UTR. During the G2/M phase of the cell cycle, the polyamines are believed to be essential for the formation of the mitotic spindle and the chromatin condensation. In addition, Pyronnet et al. *(84)* demonstrated that c-Myc mRNA, which also has been suggested to contain an IRES, was translated during the G2/M phase of the cell cycle. Thus, IRES-dependent initiation may be an important mechanism in the synthesis of some proteins during specific phases of the cell cycle (e.g., mitosis) when cap-dependent protein synthesis is inhibited. However, in spite of the vast experimental data supporting the existence of IRES in mammalian mRNAs, this notion is not without controversy *(86–90)*.

ODC expression is highly regulated by the polyamines *(3,9)*. A major part of this control is carried out at a posttranslational level. However, part of the feedback regulation of ODC exerted by the polyamines appears to be at the translational level *(61,91)*. ODC synthesis is downregulated in the presence of an excess of polyamines and upregulated in situations of polyamine deficiency. The cellular ODC mRNA level, on the other hand, is not affected by changes in cellular polyamine content, indicating a translational mechanism.

As described earlier, ODC mRNA has a long G/C rich 5′ UTR, which may be involved in the translational control of the enzyme. However, results from experiments in which the 5′ UTR was added to the mRNAs of various reporter genes, as with β-galactosidase, CAT, and luciferase, indicated that the 5′ UTR was not important for the polyamine-mediated regulation of ODC synthesis *(57,62)*. Moreover, no difference in feedback regulation of ODC synthesis existed between Chinese hamster ovary cells expressing the full-length ODC mRNA and those expressing an ODC mRNA lacking the major part of the 5′ UTR, demonstrating that the polyamine-mediated translational control of ODC is not dependent on the 5′ UTR of the mRNA *(92)*.

Exposure of cells to hypotonic stress has been shown to strongly induce ODC activity *(52–54)*. The increase, which is very fast, occurs independent of a suppression of general protein synthesis. Results from various studies indicate that the increase in ODC activity is caused by both a decrease in degradation and an increase in synthesis. The level of ODC mRNA does not usually change, indicating that the change in ODC synthesis is mainly an increase in translation, rather than a change in transcription. Using a series of stable transfectants of Chinese hamster ovary cells expressing ODC mRNAs with various truncations in the 5′ and 3′ UTRs it was demonstrated that the hypotonic induction of ODC mRNA translation was highly dependent on the presence of the 3′ UTR, but not of the 5′ UTR *(93)*. Cells expressing ODC mRNAs with various deletions in the 5′ UTR still induced ODC, whereas cells expressing ODC mRNAs without the 3′ UTR did not, or only slightly, induce ODC after exposure to hypotonic stress. Thus the 3′ UTR of ODC mRNA appears to affect the translation of the message in some way. Interestingly, the 3′ UTR of ODC mRNA has been shown to partially neutralize the inhibition exerted by the 5′ UTR, indicating that this part of the ODC mRNA may play a role in the translational control of the enzyme by interacting with the 5′ UTR *(57,59)*. However, it appears that such an interaction is not a prerequisite for the hypotonic induction of ODC expression since the induction occurred also in cells expressing ODC mRNA without the 5′ UTR *(93)*.

That the 3′ UTR of an mRNA also may be participating in the regulation of translation has attracted increased attention during recent years. A number of protein factors and sequence elements associated with the effects on translation mediated by the 3′ UTR have been described *(94–96)*. However, the exact mechanism by which the 3′ UTR of ODC mRNA affects the translation of the message during hypotonic stress remains to be established.

4. Posttranslational Regulation

Mammalian ODC has a very fast turnover *(9,10)*. Although the half-life of ODC is usually something between 30 and 60 min, it may be as short as a few minutes. The turnover of mammalian ODC is affected by the cellular polyamine levels *(9,10,97)*. In the presence of polyamine excess, there is a marked increase in the degradation rate of ODC, whereas when cells are depleted of their polyamines, the degradation of ODC is decreased.

As with other cellular proteins with a fast turnover, ODC is degraded by the 26S proteasome *(98)*. However, in contrast to the degradation of most other proteins by the proteasome, the degradation of mammalian ODC is not induced by ubiquitination *(98)*. ODC was the first example of a nonubiquitinated protein being degraded by the 26S proteasome. Instead, the degradation of ODC by this proteolytic system is induced by the strong binding of a specific protein, termed antizyme (AZ), to the enzyme *(9,10,97)*. The binding of AZ to ODC inhibits the enzyme activity, most likely because the binding of AZ, which occurs at the monomer level, hampers the formation of the homodimer. Furthermore, the binding of AZ to ODC seems to affect the monomer structure in such a way that the C-terminal part is exposed *(9,10)*. The C-terminal part of ODC has been shown to be essential for the rapid degradation of the enzyme *(99)*. The polyamines induce the degradation of ODC by affecting the synthesis of AZ, which is dependent on a unique mechanism involving ribosomal frameshifting *(100,101)*. The AZ mRNA has two major reading frames, which both have to be translated for the synthesis of the entire protein. Conventional translation of AZ mRNA arrests at a premature stop signal in the first reading frame. However, in the presence of polyamines, there is a +1 frameshift occurring at the premature termination codon, which results in a continued translation of the second reading frame and the production of full-length AZ (Fig. 3). This mechanism is unique and so far no other examples of mammalian ribosomal frameshifting are known. The mechanism is highly dependent on polyamines. In the absence or in the presence of low concentrations of polyamines, the number of ribosomes reading both frames is extremely low *(100,101)*. Thus polyamines control the turnover rate of ODC by affecting the synthesis of AZ. When there is an excess of polyamines in the cell, the synthesis of AZ is increased, resulting in a stimulation of ODC degradation. On the other hand, when cells are depleted of their polyamines, the synthesis of AZ is low resulting in a decreased turnover of ODC. Recently, it was demonstrated that in yeast, polyamines regulate ODC turnover by reducing the degradation of AZ, which occurs by the proteasome in an ubiquitin-dependent manner, in addition to inducing its expression (by stimulating frame shifting) *(102)*. However, it is not known yet whether the same mechanism also occurs in mammalian cells. Interestingly, the mammalian genome contains several active AZ genes

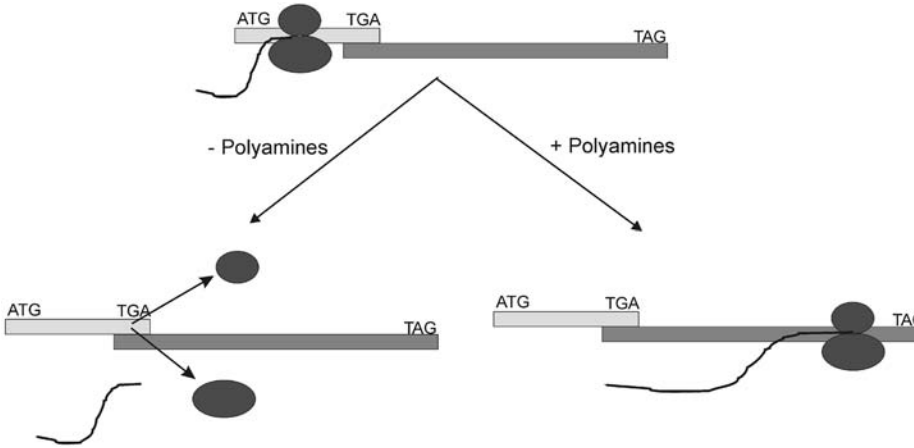

Fig. 3. Synthesis of ODC antizyme (AZ). Polyamines stimulate a +1 frameshift, resulting in the synthesis of full-length AZ. In the absence of polyamines translation is terminated at the in-frame stop codon, giving rise to a nonfunctional truncated AZ.

(103,104). Whether all these genes are involved in the regulation of ODC degradation or whether they have some other functions is not known yet.

Interestingly, ODC from the trypanosomatid *Crithidia fasciculata*, a monogenetic parasite that colonize the digestive tract of flies, still turns over rapidly in the parasite as well as when expressed in mammalian cells, even though it lacks the region corresponding to the C-terminal part of mammalian ODC *(105,106)*. *C. fasciculata* is the first protozoan organism shown to have an ODC with a short half-life. Thus *C. fasciculata* ODC must contain other signals that target the enzyme for rapid degradation also in mammalian cells. As with the mammalian ODC, *C. fasciculata* ODC seems to be degraded the 26S proteasome *(106)*. However, in contrast to mammalian ODC, *C. fasciculata* ODC is not downregulated by polyamines when expressed in mammalian cells, suggesting that the degradation is not dependent on AZ. Instead, the rapid degradation of this ODC may be mediated by ubiquitination. If so, this would be the first demonstration of an ODC being ubiquitinated.

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References

1. Tabor, H. and Tabor, C. W. (1984) Polyamines. *Ann. Rev. Biochem.* **53,** 749–790.
2. Pegg, A. E. (1988) Polyamine metabolism and its importance in neoplastic growth and as a target for chemotherapy. *Cancer Res.* **48,** 759–774.
3. Heby, O. and Persson, L. (1990) Molecular genetics of polyamine synthesis in eukaryotic cells. *Trends Biochem. Sci.* **15,** 153–158.

4. Jänne, J., Alhonen, L., and Leinonen, P. (1991) Polyamines: From molecular biology to clinical applications. *Ann. Med.* **23,** 241–259.
5. Marton, L. J. and Pegg, A. E. (1995) Polyamines as targets for therapeutic intervention. *Annu. Rev. Pharmacol. Toxicol.* **35,** 55–91.
6. Wallace, H. M., Fraser, A. V., and Hughes, A. (2003) A perspective of polyamine metabolism. *Biochem. J.* **376,** 1–14.
7. Tome, M. E., Fiser, S. M., Payne, C. M., and Gerner, E. W. (1997) Excess putrescine accumulation inhibits the formation of modified eukaryotic initiation factor 5A (eIF-5A) and induces apoptosis. *Biochem. J.* **328,** 847–854.
8. McCann, P. P. and Pegg, A. E. (1992) Ornithine decarboxylase as an enzyme target for therapy. *Pharmacol. Ther.* **54,** 195–215.
9. Hayashi, S., Murakami, Y., and Matsufuji, S. (1996) Ornithine decarboxylase antizyme: A novel type of regulatory protein. *Trends Biochem. Sci.* **21,** 27–30.
10. Coffino, P. (2001) Regulation of cellular polyamines by antizyme. *Nat. Rev. Mol. Cell. Biol.* **2,** 188–194.
11. Watanabe, S., Kusama-Eguchi, K., Kobayashi, H., and Igarashi, K. (1991) Estimation of polyamine binding to macromolecules and ATP in bovine lymphocytes and rat liver. *J. Biol. Chem.* **266,** 20,803–20,809.
12. Katz, A. and Kahana, C. (1987) Transcriptional activation of mammalian ornithine decarboxylase during stimulated growth. *Mol. Cell. Biol.* **7,** 2641–2643.
13. Olson, E. N. and Spizz, G. (1986) Mitogens and protein synthesis inhibitors induce ornithine decarboxylase gene transcription through separate mechanisms in the BC3H1 muscle cell line. *Mol. Cell. Biol.* **6,** 2792–2799.
14. Wallon, U. M., Persson, L., and Heby, O. (1995) Regulation of ornithine decarboxylase during cell growth. Changes in the stability and translatability of the mRNA, and in the turnover of the protein. *Mol. Cell Biochem.* **146,** 39–44.
15. Law, G. L., Li, R.-S., and Morris, D. R. (1996) Transcriptional control of the ODC gene. In: *Polyamines: Regulation and Molecular Interaction* (Casero, Jr., R. A., ed.), R.G. Landes Company, Austin, TX pp. 5–26.
16. Abrahamsen, M. S., Li, R.-S., Dietrich-Goetz, W., and Morris, D. R. (1992) Multiple DNA elements responsible for transcriptional regulation of the ornithine decarboxylase gene by protein kinase A. *J. Biol. Chem.* **267,** 18,866–18,873.
17. Verma, A. K. (1988) Inhibition of phorbol ester-induced ornithine decarboxylase gene transcription by retinoic acid: a possible mechanism of antitumor promoting activity of retinoids. *Prog. Clin. Biol. Res.* **259,** 245–260.
18. Verma, A. K., Hsieh, J. T., and Pong, R. C. (1988) Mechanisms involved in ornithine decarboxylase induction by 12-O-tetradecanoylphorbol-13-acetate, a potent mouse skin tumor promoter and an activator of protein kinase C. *Adv. Exp. Med. Biol.* **250,** 273–290.
19. O'Brien, T. G., Megosh, L. C., Gilliard, G., and Soler, A. P. (1997) Ornithine decarboxylase overexpression is a sufficient condition for tumor promotion in mouse skin. *Cancer Res.* **57,** 2630–2637.
20. Megosh, L., Gilmour, S. K., Rosson, D., et al. (1995) Increased frequency of spontaneous skin tumors in transgenic mice which overexpress ornithine decarboxylase. *Cancer Res.* **55,** 4205–4209.
21. Guo, Y., Cleveland, J. L., and O'Brien, T. G. (2005) Haploinsufficiency for odc modifies mouse skin tumor susceptibility. *Cancer Res.* **65,** 1146–1149.
22. Auvinen, M., Paasinen, A., Andersson, L. C., and Hölttä, E. (1992) Ornithine decarboxylase activity is critical for cell transformation. *Nature* **360,** 355–358.

23. Auvinen, M., Laine, A., Paasinen-Sohns, A., et al. (1997) Human ornithine decarboxylase-overproducing NIH3T3 cells induce rapidly growing, highly vascularized tumors in nude mice. *Cancer Res.* **57,** 3016–3025.
24. Nilsson, J. A. and Cleveland, J. L. (2003) Myc pathways provoking cell suicide and cancer. *Oncogene* **22,** 9007–9021.
25. Prendergast, G. C., Lawe, D., and Ziff, E. B. (1991) Association of Myn, the murine homolog of max, with c-Myc stimulates methylation-sensitive DNA binding and ras cotransformation. *Cell* **65,** 395–407.
26. Blackwell, T. K., Huang, J., Ma, A., et al. (1993) Binding of myc proteins to canonical and noncanonical DNA sequences. *Mol. Cell. Biol.* **13,** 5216–5224.
27. Bello-Fernandez, C. and Cleveland, J. L. (1992) c-*myc* Transactivates the ornithine decarboxylase gene. *Curr. Top. Microbiol. Immunol.* **182,** 445–452.
28. Bello-Fernandez, C., Packham, G., and Cleveland, J. L. (1993) The ornithine decarboxylase gene is a transcriptional target of c-Myc. *Proc. Natl. Acad. Sci. USA* **90,** 7804–7808.
29. Tobias, K. E., Shor, J., and Kahana, C. (1995) C-Myc and Max transregulate the mouse ornithine decarboxylase promoter through interaction with two downstream CACGTG motifs. *Oncogene* **11,** 1721–1727.
30. Packham, G., Bello-Fernandez, C., and Cleveland, J. L. (1994) Position and orientation independent transactivation by c-Myc. *Cell Mol. Biol. Res.* **40,** 699–706.
31. Nilsson, J. A., Maclean, K. H., Keller, U. B., Pendeville, H., Baudino, T. A., and Cleveland, J. L. (2004) Mnt loss triggers Myc transcription targets, proliferation, apoptosis, and transformation. *Mol. Cell. Biol.* **24,** 1560–1569.
32. Packham, G. and Cleveland, J. L. (1995) c-Myc and apoptosis. *Biochim. Biophys. Acta* **1242,** 11–28.
33. Packham, G., Porter, C. W., and Cleveland, J. L. (1996) C-Myc induces apoptosis and cell cycle progression by separable, yet overlapping, pathways. *Oncogene* **13,** 461–469.
34. Packham, G. and Cleveland, J. L. (1994) Ornithine decarboxylase is a mediator of c-Myc-induced apoptosis. *Mol. Cell. Biol.* **14,** 5741–5747.
35. Packham, G. and Cleveland, J. L. (1997) Induction of ornithine decarboxylase by IL-3 is mediated by sequential c-Myc-independent and c-Myc-dependent pathways. *Oncogene* **15,** 1219–1232.
36. Iwamoto, M., Ahnen, D. J., Franklin, W. A., and Maltzman, T. H. (2000) Expression of beta-catenin and full-length APC protein in normal and neoplastic colonic tissues. *Carcinogenesis* **21,** 1935–1940.
37. Groden, J., Thliveris, A., Samowitz, W., et al. (1991) Identification and characterization of the familial adenomatous polyposis coli gene. *Cell* **66,** 589–600.
38. Kinzler, K. W., Nilbert, M. C., Su, L. K., et al. (1991) Identification of FAP locus genes from chromosome 5q21. *Science* **253,** 661–665.
39. He, T. C., Sparks, A. B., Rago, C., et al. (1998) Identification of c-MYC as a target of the APC pathway. *Science* **281,** 1509–1512.
40. Meyskens, F. L., Jr. and Gerner, E. W. (1999) Development of difluoromethylornithine (DFMO) as a chemoprevention agent. *Clin. Cancer Res.* **5,** 945–951.
41. Erdman, S. H., Ignatenko, N. A., Powell, M. B., et al. (1999) APC-dependent changes in expression of genes influencing polyamine metabolism, and consequences for gastrointestinal carcinogenesis, in the Min mouse. *Carcinogenesis* **20,** 1709–1713.
42. Giardiello, F. M., Hamilton, S. R., Krush, A. J., et al. (1993) Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. *N. Engl. J. Med.* **328,** 1313–1316.

43. Walhout, A. J. M., Gubbels, J. M., Bernards, R., Van der Vliet, P. C., and Timmers, H. T. M. (1997) c-Myc/Max heterodimers bind cooperatively to the E-box sequences located in the first intron of the rat ornithine decarboxylase (ODC) gene. *Nucleic Acids Res.* **25,** 1493–1501.
44. Guo, Y., Harris, R. B., Rosson, D., Boorman, D., and O'Brien, T. G. (2000) Functional analysis of human ornithine decarboxylase alleles. *Cancer Res.* **60,** 6314–6317.
45. Martinez, M. E., O'Brien, T. G., Fultz, K. E., et al. (2003) Pronounced reduction in adenoma recurrence associated with aspirin use and a polymorphism in the ornithine decarboxylase gene. *Proc. Natl. Acad. Sci. USA* **100,** 7859–7864.
46. Steinbach, G., Lynch, P. M., Phillips, R. K., et al. (2000) The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. *N. Engl. J. Med.* **342,** 1946–1952.
47. Thun, M. J., Henley, S. J., and Patrono, C. (2002) Nonsteroidal anti-inflammatory drugs as anticancer agents: Mechanistic, pharmacologic, and clinical issues. *J. Natl. Cancer Inst.* **94,** 252–266.
48. Gerner, E. W., and Meyskens, F. L., Jr. (2004) Polyamines and cancer: Old molecules, new understanding. *Nat. Rev. Cancer* **4,** 781–792.
49. White, M. W., Kameji, T., Pegg, A. E., and Morris, D. R. (1987) Increased efficiency of translation of ornithine decarboxylase mRNA in mitogen-activated lymphocytes. *Eur J. Biochem.* **170,** 87–92.
50. Fredlund, J. O., Johansson, M. C., Dahlberg, E., and Oredsson, S. M. (1995) Ornithine decarboxylase and S-adenosylmethionine decarboxylase expression during the cell cycle of Chinese hamster ovary cells. *Exp. Cell Res.* **216,** 86–92.
51. Persson, L., Svensson, F., and Lövkvist Wallström, E. (1996) Regulation of polyamine metabolism. In: *Polyamines in Cancer: Basic Mechanisms and Clinical Approaches* (Nishioka, K., ed.), R.G. Landes Company, Austin, TX pp. 19–43.
52. Poulin, R., and Pegg, A. E. (1990) Regulation of ornithine decarboxylase expression by anisosmotic shock in difluoromethylornithine-resistant L1210 cells. *J. Biol. Chem.* **265,** 4025–4032.
53. Lundgren, D. W. (1992) Effect of hypotonic stress on ornithine decarboxylase mRNA expression in cultured cells. *J. Biol. Chem.* **267,** 6841–6847.
54. Lövkvist-Wallström, E., Stjernborg-Ulvsbäck, L., Scheffler, I. E., and Persson, L. (1995) Regulation of mammalian ornithine decarboxylase—Studies on the induction of the enzyme by hypotonic stress. *Eur J. Biochem.* **231,** 40–44.
55. Kozak, M. (1987) An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res.* **15,** 8125–8148.
56. Brabant, M., McConlogue, L., van Daalen Wetters, T., and Coffino, P. (1988) Mouse ornithine decarboxylase gene: cloning, structure, and expression. *Proc. Natl. Acad. Sci. USA* **85,** 2200–2204.
57. Grens, A. and Scheffler, I. E. (1990) The 5′- and 3′-untranslated regions of ornithine decarboxylase mRNA affect the translational efficiency. *J. Biol. Chem.* **265,** 11,810–11,816.
58. Manzella, J. M. and Blackshear, P. J. (1990) Regulation of rat ornithine decarboxylase mRNA translation by its 5′-untranslated region. *J. Biol. Chem.* **265,** 11,817–11,822.
59. Lorenzini, E. C. and Scheffler, I. E. (1997) Co-operation of the 5′ and 3′ untranslated regions of ornithine decarboxylase mRNA and inhibitory role of its 3′ untranslated region in regulating the translational efficiency of hybrid RNA species via cellular factor(s). *Biochem. J.* **326,** 361–367.
60. Manzella, J. M. and Blackshear, P. J. (1992) Specific protein binding to a conserved region of the ornithine decarboxylase mRNA 5′-untranslated region. *J. Biol. Chem.* **267,** 7077–7082.

61. Holm, I., Persson, L., Stjernborg, L., Thorsson, L., and Heby, O. (1989) Feedback control of ornithine decarboxylase expression by polyamines. Analysis of ornithine decarboxylase mRNA distribution in polysome profiles and of translation of this mRNA in vitro. *Biochem. J*. **258,** 343–350.
62. van Daalen Wetters, T., Macrae, M., Brabant, M., Sittler, A., and Coffino, P. (1989) Polyamine-mediated regulation of mouse ornithine decarboxylase is post-translational. *Mol. Cell. Biol.* **9,** 5484–5490.
63. Kahana, C. and Nathans, D. (1985) Nucleotide sequence of murine ornithine decarboxylase mRNA. *Proc. Natl. Acad. Sci. USA* **82,** 1673–1677.
64. McKendrick, L., Pain, V. M., and Morley, S. J. (1999) Translation initiation factor 4E. *Int. J. Biochem. Cell Biol.* **31,** 31–35.
65. Koromilas, A. E., Lazaris-Karatzas, A., and Sonenberg, N. (1992) mRNAs containing extensive secondary structure in their 5′ non-coding region translate efficiently in cells overexpressing initiation factor eIF-4E. *EMBO J.* **11,** 4153–4158.
66. Kozak, M. (1999) Initiation of translation in prokaryotes and eukaryotes. *Gene* **234,** 187–208.
67. Kozak, M. (1989) Circumstances and mechanisms of inhibition of translation by secondary structure in eucaryotic mRNAs. *Mol. Cell. Biol*. **9,** 5134–5142.
68. Pain, V. M. (1996) Initiation of protein synthesis in eukaryotic cells. *Eur J. Biochem.* **236,** 747–771.
69. Lynch, M., Fitzgerald, C., Johnston, K. A., Wang, S., and Schmidt, E. V. (2004) Activated eIF4E-binding protein slows G1 progression and blocks transformation by c-myc without inhibiting cell growth. *J. Biol. Chem.* **279,** 3327–3339.
70. Polunovsky, V. A., Rosenwald, I. B., Tan, A. T., et al. (1996) Translational control of programmed cell death: Eukaryotic translation initiation factor 4E blocks apoptosis in growth-factor-restricted fibroblasts with physiologically expressed or deregulated Myc. *Mol. Cell. Biol*. **16,** 6573–6581.
71. Raught, B. and Gingras, A. C. (1999) eIF4E activity is regulated at multiple levels. *Int. J. Biochem. Cell Biol.* **31,** 43–57.
72. Lazaris-Karatzas, A., Montine, K. S., and Sonenberg, N. (1990) Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5′ cap. *Nature* **345,** 544–547.
73. Zimmer, S. G., DeBenedetti, A., and Graff, J. R. (2000) Translational control of malignancy: the mRNA cap-binding protein, eIF-4E, as a central regulator of tumor formation, growth, invasion and metastasis. *Anticancer Res.* **20,** 1343–1351.
74. Rousseau, D., Kaspar, R., Rosenwald, I., Gehrke, L., and Sonenberg, N. (1996) Translation initiation of ornithine decarboxylase and nucleocytoplasmic transport of cyclin D1 mRNA are increased in cells overexpressing eukaryotic initiation factor 4E. *Proc. Natl. Acad. Sci. USA* **93,** 1065–1070.
75. Shantz, L. M., Hu, R. H., and Pegg, A. E. (1996) Regulation of ornithine decarboxylase in a transformed cell line that overexpresses translation initiation factor eIF-4E. *Cancer Res.* **56,** 3265–3269.
76. Graff, J. R., De Benedetti, A., Olson, J. W., Tamez, P., Casero, R. A., Jr., and Zimmer, S. G. (1997) Translation of ODC mRNA and polyamine transport are suppressed in *ras*-transformed CREF cells by depleting translation initiation factor 4E. *Biochem. Biophys. Res. Commun.* **240,** 15–20.
77. Shantz, L. M. and Pegg, A. E. (1994) Overproduction of ornithine decarboxylase caused by relief of translational repression is associated with neoplastic transformation. *Cancer Res.* **54,** 2313–2316.

78. Shantz, L. M., Coleman, C. S., and Pegg, A. E. (1996) Expression of an ornithine decarboxylase dominant-negative mutant reverses eukaryotic initiation factor 4E-induced cell transformation. *Cancer Res.* **56,** 5136–5140.
79. Martinez-Salas, E. (1999) Internal ribosome entry site biology and its use in expression vectors. *Curr. Opin. Biotechnol.* **10,** 458–464.
80. Sachs, A. B. (2000) Cell cycle-dependent translation initiation: IRES elements prevail. *Cell* **101,** 243–245.
81. Pelletier, J. and Sonenberg, N. (1988) Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. *Nature* **334,** 320–325.
82. Sonenberg, N. (1990) Measures and counter measures in the modulation of initiation factor activities by viruses. *New Biol.* **2,** 402–409.
83. van der Velden, A. W., Thomas, A. A. (1999) The role of the 5′ untranslated region of an mRNA in translation regulation during development. *Int. J. Biochem. Cell Biol.* **31,** 87–106.
84. Pyronnet, S., Pradayrol, L., and Sonenberg, N. (2000) A cell cycle-dependent internal ribosome entry site. *Mol. Cell* **5,** 607–616.
85. Heby, O., Gray, J. W., Lindl, P. A., Marton, L. J., and Wilson, C. B. (1976) Changes in L-ornithine decarboxylase activity during the cell cycle. *Biochem. Biophys. Res. Commun.* **71,** 99–105.
86. Kozak, M. (2001) New ways of initiating translation in eukaryotes? *Mol. Cell. Biol.* **21,** 1899–1907.
87. Schneider, R., Agol, V. I., Andino, R., et al. (2001) New ways of initiating translation in eukaryotes. *Mol. Cell. Biol.* **21,** 8238–8246.
88. Kozak, M. (2003) Alternative ways to think about mRNA sequences and proteins that appear to promote internal initiation of translation. *Gene* **318,** 1–23.
89. Van Eden, M. E., Byrd, M. P., Sherrill, K. W., and Lloyd, R. E. (2004) Demonstrating internal ribosome entry sites in eukaryotic mRNAs using stringent RNA test procedures. *RNA* **10,** 720–730.
90. Komar, A. A. and Hatzoglou, M. (2005) Internal ribosome entry sites in cellular mRNAs: The mystery of their existence. *J. Biol. Chem.* **280,** 23,425–23,428.
91. Persson, L., Holm, I., and Heby, O. (1988) Regulation of ornithine decarboxylase mRNA translation by polyamines. Studies using a cell-free system and a cell line with an amplified ornithine decarboxylase gene. *J. Biol. Chem.* **263,** 3528–3533.
92. Lövkvist Wallström, E. and Persson, L. (1999) No role of the 5′ untranslated region of ornithine decarboxylase mRNA in the feedback control of the enzyme. *Mol. Cell. Biochem.* **197,** 71–78.
93. Lövkvist, W. E., Takao, K., Wendt, A., Vargiu, C., Yin, H., and Persson, L. (2001) Importance of the 3′ untranslated region of ornithine decarboxylase mRNA in the translational regulation of the enzyme. *Biochem. J.* **356,** 627–634.
94. Black, B. L., Lu, J., and Olson, E. N. (1997) The MEF2A 3′ untranslated region functions as a cis-acting translational repressor. *Mol. Cell. Biol.* **17,** 2756–2763.
95. Piecyk, M., Wax, S., Beck, A. R., et al. (2000) TIA-1 is a translational silencer that selectively regulates the expression of TNF-alpha. *EMBO J.* **19,** 4154–4163.
96. Mbella, E. G., Bertrand, S., Huez, G., and Octave, J. N. (2000) A GG nucleotide sequence of the 3′ untranslated region of amyloid precursor protein mRNA plays a key role in the regulation of translation and the binding of proteins. *Mol. Cell. Biol.* **20,** 4572–4579.
97. Hayashi, S. and Murakami, Y. (1995) Rapid and regulated degradation of ornithine decarboxylase. *Biochem. J.* **306,** 1–10.
98. Murakami, Y., Matsufuji, S., Kameji, T., et al. (1992) Ornithine decarboxylase is degraded by the 26S proteasome without ubiquitination. *Nature* **360,** 597–599.

99. Ghoda, L., van Daalen Wetters, T., Macrae, M., Ascherman, D., and Coffino, P. (1989) Prevention of rapid intracellular degradation of ODC by a carboxyl-terminal truncation. *Science* **243,** 1493–1495.
100. Rom, E. and Kahana, C. (1994) Polyamines regulate the expression of ornithine decarboxylase antizyme *in vitro* by inducing ribosomal frame-shifting. *Proc. Natl. Acad. Sci. USA* **91,** 3959–3963.
101. Matsufuji, S., Matsufuji, T., Miyazaki, Y., et al. (1995) Autoregulatory frameshifting in decoding mammalian ornithine decarboxylase antizyme. *Cell* **80,** 51–60.
102. Palanimurugan, R., Scheel, H., Hofmann, K., and Dohmen, R. J. (2004) Polyamines regulate their synthesis by inducing expression and blocking degradation of ODC antizyme. *EMBO J.* **23,** 4857–4867.
103. Ivanov, I. P., Gesteland, R. F., and Atkins, J. F. (1998) A second mammalian antizyme: conservation of programmed ribosomal frameshifting. *Genomics* **52,** 119–129.
104. Ivanov, I. P., Rohrwasser, A., Terreros, D. A., Gesteland, R. F., and Atkins, J. F. (2000) From the cover: discovery of a spermatogenesis stage-specific ornithine decarboxylase antizyme: Antizyme 3 [In Process Citation]. *Proc. Natl. Acad. Sci. USA* **97,** 4808–4813.
105. Svensson, F., Ceriani, C., Lövkvist Wallström, E., et al. (1997) Cloning of a trypanosomatid gene coding for an ornithine decarboxylase that is metabolically unstable even though it lacks the C-terminal degradation domain. *Proc. Natl. Acad. Sci. USA* **94,** 397–402.
106. Nasizadeh, S., Jeppsson, A., and Persson, L. (2003) Proteasomal degradation of a trypanosomal ornithine decarboxylase. *Cell. Physiol. Biochem.* **13,** 321–328.

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Evidence for a Multistep Model for Eukaryotic Polyamine Transport

Richard Poulin, Denis Soulet, Bruno Gagnon, Serge Rivest, and Marie Audette

1. Introduction

One of the most intriguing aspects of polyamine biology is the considerable diversity of their functions in the cell. The involvement of polyamines in such a multiplicity of parallel activities obviously requires mechanisms for the regulation of their concentrations in the various intracellular compartments. Much progress has been made in our understanding of polyamine homeostasis, and it is now clear that antizymes (AZ) are major players in regulating the size of cellular polyamine pools through the feedback inhibition exerted by these proteins on ornithine decarboxylase (ODC) activity and levels, and on polyamine uptake activity *(1)*. However, our current view of how polyamines are distributed throughout the cytoplasm and nucleus after their synthesis is severely limited. The problem of polyamine microcompartmentalization is especially important in eukaryotic cells, where polyamines are expected to simultaneously act in the cytosol (e.g., in ribosomes), in close vicinity of the plasma membrane (e.g., ion channel gating), and in membrane-bound organelles (e.g., nucleus, mitochondria).

Many observations already support the notion that polyamines are not uniformly distributed inside eukaryotic ells. For instance, lower fungi clearly store a major fraction of the spermidine pool inside the vacuole (*see* Chapter 25). Subcellular polyamine transport systems have been demonstrated in isolated mitochondria *(2)* and synaptic vesicles *(3)*. Moreover, a substantial fraction of AZ 1 and ODC, which have usually been considered as cytosolic proteins, has been shown to localize in the nucleus under certain physiological conditions *(4)*. The most compelling evidence that the bulk of cellular polyamines most likely exists in an inert, heterogeneously distributed form, comes from a comparison between their total intracellular concentration as extrapolated to total cell volume, and their thermodynamic activity as predicted from their binding affinity for DNA, RNA, phospholipids, and adenosine triphosphate (ATP) *(5)*.

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Under physiological conditions, such calculations predict that most spermidine and spermine would exist as complexes with RNA, and thus be thermodynamically inactive. Such physicochemical sequestration of intracellular polyamines would help to account for the fact that upregulation of AZ 1 *(6)* or spermidine/spermine N^1-acetyltransferase (SSAT) expression *(7)* induced by exogenous polyamines is often observed without notable changes in the polyamine pool size. The requirement for intracellular spermidine for SSAT induction by certain chemical or physical insults in the absence of exogenous polyamines was proposed nearly 20-yr ago to reflect the disruption by such stimuli of a pre-existing subcellular microcompartmentalization of polyamines *(8)*. Further indirect support for the quantitative importance of polyamine sequestration comes from the observation that the often large spermine pool left in cells treated with the ODC inhibitor, α-difluoromethylornithine (DFMO), is ineffective for the induction of SSAT *(7)* or for preventing AZ depletion *(9)*, whereas exogenous spermine is a strong inducer of both proteins *(10,11)*.

Thus there is strong circumstantial evidence for a very heterogeneous distribution of polyamines inside mammalian cells and for a differential compartmentalization of endogenous and exogenous polyamines in the mammalian cell. This chapter outlines a new paradigm derived from recent work in our laboratory that has radically changed our view on the organization of the mammalian polyamine transport system and the subcellular distribution of polyamines.

2. Physiological and Biochemical Characterization of Polyamine Transport and Compartmentalization in Mammalian Cells

Before discussing the recent fluorometric studies of polyamine internalization, it is useful to summarize previous studies on the properties of polyamine transport in mammalian cells and on the subcellular compartmentalization of polyamines. Unfortunately, even at this stage, no molecular characterization of a polyamine transporter is yet available for animal species, although the very recent description of a polyamine permease in the parasite *Leishmania major (12)* may constitute a major step toward this goal. Our knowledge on the properties of mammalian polyamine carriers still relies on their physiological properties at the whole cell level.

2.1. Polyamine Transport Systems

2.1.1. Importance of Polyamine Transport in Mammals

Diamine and polyamine transport activity has been detected in virtually all tissue and cell types *(13–15)*. A higher polyamine uptake activity is frequently correlated with active cell proliferation and accompanies transformation to a tumorigenic state. Thus mammalian polyamine transport generally increases in concert with ODC on mitogenic stimulation by either growth factors, hormones, or oncogenic transformation *(15,16)*. It has become increasingly clear that plasma and circulating polyamines derived from the diet, from the gastrointestinal microflora, and from excretion from peripheral tissues play a major role in the homeostasis of these compounds in the whole organism *(13,15)*. Thus influx of exogenous polyamines complements intracellular biosynthesis from amino acid precursors and represents a component that must be

repressed in therapeutic strategies based on polyamine depletion, or that may be efficiently targeted in those relying on cytotoxic polyamine analogs *(15)*.

2.1.2. Biochemical Properties of Mammalian Polyamine Transporters

There is no clear consensus on the actual number of different diamine and polyamine transport systems found in mammals. A few reports have presented evidence favoring the coexistence of at least two types of carriers: a putrescine-preferential system that is strongly inhibited by spermidine and spermine and a spermidine/spermine-preferential system that is weakly inhibited by putrescine *(17)*. On the other hand, an alternative view has been proposed for a common transporter accommodating both diamines and polyamines, in which the nonreciprocal competition observed between the various substrates of that carrier would reflect their use of different subsets of binding sites *(16)*. Another controversial issue is whether mammalian polyamine transport is Na^+ dependent or not. Some studies have suggested that the uptake of putrescine, and to a lesser degree, spermidine, is a Na^+-dependent process, whereas spermine transport is Na^+ independent *(18)*. On the other hand, other groups came to the conclusion that transport of all three natural polyamines depends on membrane potential, but does not require Na^+ *(19,20)*, which inhibits polyamine transport *(21)*. A thorough review of the relevant literature indicates that in most cases, an assignment of a Na^+ dependence was made by substituting a cation, such as K^+, Li^+, or cholinium, which inhibit polyamine transport even more than Na^+ *(21)*.

Quite remarkably, the mammalian transport system has an absolute requirement for Ca^{2+}, Mg^{2+}, or Mn^{2+} at the exofacial side of the plasma membrane *(21,22)*, suggesting that the carrier or a regulatory protein thereof has a divalent metal binding site essential for polyamine uptake activity. Such binding sites might include exofacial carboxylic residues outside the substrate binding site of the carrier that are essential for transport activity *(23)*. Thiol groups that are essential for substrate translocation are also present in the polyamine carrier(s) *(13)*. Furthermore, polyamine transport is sharply inhibited at an extracellular pH less than 6.5 *(21)*, suggesting the presence of an essential titratable residue in the carrier.

The substrate specificity of the mammalian polyamine transport system is rather wide by usual standards, and considerable versatility in the nature and size of the backbone and substituents can be introduced without compromising substrate affinity nor specific recognition by the polyamine transport system. Even bulky hydrophobic substituents larger than the polyamine backbone itself do not compromise the specific uptake of polyamine derivatives *(24–26)*. Nevertheless important features can been identified, such as the detrimental effect of carboxylic groups on substrate affinity and the requirement for at least two positively charged centers distant by at least three methylene groups *(15,16,24,27)*. The unusual plasticity of substrate use by the mammalian polyamine transport system reminds that described for transporters belonging to the ATP-binding cassette family such as the P-glycoprotein, which interacts with a wide variety of substrates through multiple binding sites within its transmembrane domains *(28)*. The versatility of the polyamine transport system has been a useful characteristic for the inclusion of fluorescent side chains for the design of transport probes *(25,26,29)* or cytotoxic conjugates *(27)*.

2.1.3. Feedback Regulation of the Mammalian Polyamine Transport System

An important feature of the mammalian polyamine transport system is the modulation of its activity by intracellular polyamines. It had been noted quite early that the depletion of putrescine and spermidine induced by DFMO strongly upregulates polyamine uptake activity *(13,15,16)*. This upregulation results from an increase in the maximal velocity of the rate of polyamine influx and in the maximal level of substrate accumulation. Conversely, the intracellular accumulation of exogenous polyamines rapidly suppresses net uptake activity, an effect that requires active protein synthesis *(16)*. The factors responsible for this feedback inhibition are AZ 1 and 2, which also promote rapid ODC degradation *(1)*. The synthesis of active AZ is induced by increases in polyamine concentrations via a ribosomal frameshifting event (*see* Chapter 26), and thus play a central role in polyamine homeostasis by limiting the rate of polyamine biosynthesis and transport in a coordinated fashion *(1)*. The nature of the interaction between AZ and polyamine transporters is as yet unknown, but increasing evidence that AZ are involved in the formation of complexes for the targeting of various proteins to the proteasome *(30)* suggest that the turnover of polyamine carriers or accessory proteins interacting with them involves the proteasome pathway. In addition to the AZ-dependent feedback repression, a second mechanism triggered by polyamine accumulation downregulates polyamine transport activity without requiring *de novo* protein synthesis *(16)*.

2.2. The Riddle of Polyamine Compartmentalization

When weighted according to cell volume, intracellular spermidine and spermine concentrations reach the order of 10^{-3} mol/L^{-1} in mammalian cells *(31)*. Such figures are often taken as evidence for the biological relevance of some actions exerted by high polyamine concentrations in cell-free systems. However, it is clear that the prevalence of millimolar concentrations of polyamines in all cell compartments would leave little room for regulatory adjustments because most of their presumptive effects on biochemical structures or reactions are observed at much lower concentrations. Moreover, there is substantial evidence that polyamines can induce apoptosis in the range of concentrations calculated assuming a uniform distribution of these compounds in the cytoplasm *(32)*. If polyamines do behave as signaling molecules, their subcellular distribution must be finely controlled and allow rapid and reversible adjustments as in the case of Ca^{2+} stores *(33)*.

A thorough discussion of the various studies on polyamine compartmentalization would require careful assessments of the different methodologies used, but would go beyond the limits of this chapter. Previous strategies used to study the microcompartmentalization of endogenous polyamines have mainly relied on subcellular fractionation, cytochemistry using the fluorescamine or *o*-phthalaldehyde reactions, or immunocytochemical detection with various antipolyamine antibodies, using various protocols for fixation and permeabilization *(34–36* and references therein). No consensus can be clearly drawn from these studies, and the localization of polyamines has been described as exclusively cytoplasmic (most often in association with ribosome-rich regions), or distributed in various ratios between the nucleus (especially with

heterochromatin) and the cytoplasm. Specific immunoreactivity was reported to markedly increase on DNase and RNase treatment *(37)*, indicating that a subset of the polyamine pool exists as complexes with nucleic acids.

Watanabe et al. *(5)* have used a different rationale to the problem of polyamine compartmentalization. The binding constants of spermidine and spermine to various DNA, RNA, ATP, and phospholipids were experimentally determined in a cell-free system under physiological concentrations of K^+ and Mg^{2+} and used to theoretically predict the steady-state concentration of the free polyamines under conditions simulating the intracellular microenvironment. The authors came to the conclusion that most intracellular spermidine and spermine exist as complexes with ribosomal RNA and transfer RNA, with DNA-bound polyamines representing only a minor fraction of the total sequestered by macromolecules.

The demonstration by different groups of shuttling of AZ 1 between the nucleus and cytoplasm *(4)* and the evidence that a subpopulation of ODC has a nuclear localization *(38)* support the view that the subcellular distribution of polyamines is still poorly understood and is likely to be more complex than previously anticipated.

3. Dissecting the Pathway of Polyamine Internalization Using Fluoroprobes

The studies described aimed at understanding the subcellular distribution of endogenous polyamines, and other groups have developed methodological strategies to visualize newly internalized polyamines. Polyamine biosynthesis is considered to occur chiefly in the cytosol, and because the simplest possible model for polyamine uptake involves the translocation of substrates from medium to the cytosol, the intracellular distribution of newly internalized polyamines and endogenous polyamines could be considered to be identical as an initial hypothesis.

3.1. Rationale for the Design of Polyamine Fluoroprobes and Initial Studies

At least four laboratories have independently developed fluorescent polyamine analogs as probes to visualize the internalization mechanism and to assess the subcellular distribution of polyamines. Either the terminal (N^1) amino group *(29,39)* or central (N^4) amino groups *(25,26,40)* of a polyamine backbone has been used for conjugating the fluorophore. It is noteworthy that the size of the fluorophore in the conjugates was substantial relative to the polyamine backbone. Except for the fluorescein-derivatized probe *(29)*, which is negatively charged at physiological pH, the fluorescent moieties added to the polyamine (e.g., BODIPY-FL) (Fig. 1A) *(26)*, *N*-methylanthranyl (Fig. 1B) *(25)*, or 9-anthracenylmethyl (Fig. 1C) *(39)* are markedly hydrophobic (Fig. 1). Preference for a more hydrophobic fluorophore was based on the assumption that only the polyamine backbone should be impermeant toward the cell membrane and limiting for translocation.

In all cases, the fluorescent polyamine conjugates were shown to be specifically transported via the mammalian polyamine transport system, as shown by competitive inhibition of probe influx by natural polyamines. Moreover, polyamine transport-deficient Chinese hamster ovary (CHO) cell mutants were completely unable to internalize the derivatives *(25,26,40)*. As a further indication that the derivatives behaved

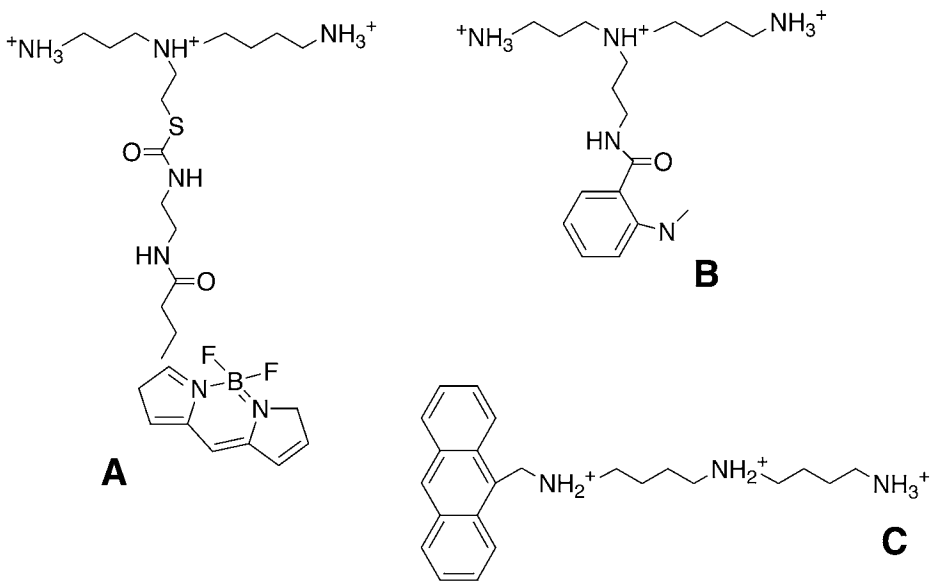

Fig. 1. Structures of three representative fluorescent polyamines used as probes of polyamine transport. **(A)** Spd-C_2-BODIPY *(26)*, **(B)** *N*-{spermidine-[N^4-(3-aminopropyl)]}anthranylamide *(25)*, **(C)** *N*-(4-aminobutyl)-*N*-anthracen-9-ylmethylbutane-1,4-diamine *(39)*.

like the parent polyamine, prior treatment with DFMO or cycloheximide (a treatment that pre-empts AZ induction and feedback transport inhibition by accumulating substrate) increased the velocity of probe transport *(26)*. Thus the novel fluorescent polyamines physiologically behaved like the parental molecules and required a functional polyamine transport system for their internalization.

Quite remarkably, a feature that was uniformly reported for all fluoroprobes studied was an exclusively cytoplasmic localization of the internalized molecules, as observed by epifluorescence microscopy or by confocal laser scanning microscopy *(25,26,29,40)*. Only extremely weak labeling of chromatin regions could be detected under any circumstances, even in mitotic cells *(25)*. However, the most unexpected finding was the observation that fluoroprobe accumulation was largely confined to vesicle-like structures *(25,26,40)*. The remarkable pattern of these polyamine-sequestering vesicles (PSVs) first led to the suggestion that the polyamine transport process was mediated by receptor-mediated endocytosis rather than classic translocation from the exo- to the endofacial sides of the plasma membrane *(25,26)*. A somewhat similar endocytic mode of polyamine uptake has been proposed to account for the role of glypican-bound heparan sulfate in spermine uptake *(41)*. In the latter model, spermine tightly complexed with heparin sulfate chains would be first internalized via endocytic caveolation of glycosylphosphatidylinositol-bound glypican-1 and then be released from the specialized endosomes according to a complex processing of heparin chains involving nitric oxide *(41)*.

3.2. Polyamine Uptake and Sequestering by Mammalian Cells: A Two-Step, Two-Compartment Process

In our initial studies with spermidine-C_2-BODIPY (Spd-C_2-BODIPY) (Fig. 1), the hypothesis that accumulation of the polyamine probe into PSVs proceeds via receptor-mediated endocytosis was initially evaluated by measuring the extent of its colocalization with fluorescent transferrin *(26)*. The extent of such colocalization was significant, but only partial, and limited to the largest PSVs. Thus PSVs did include vesicles converging with the recycling endocytosis pathway, but other structures were clearly participating to the vesicular accumulation of the polyamine probe *(26)*.

Our group has therefore proposed two different models to account for the vesicular accumulation of exogenous polyamine probes (Fig. 2). The first model involved the binding of the polyamine substrate to a cell surface receptor followed by the internalization of the resulting complex by receptor-mediated endocytosis. By analogy with the mode proposed for iron transport by DMT1 (=Slc11a2) *(42)*, polyamines would be released from the internalized receptors as a result of endosomal acidification, and subsequently exported from PSVs to the cytosol via a vesicular membrane exporter *(26,40)*. The second model involved a first step mediated by the translocation of polyamines into the cytoplasm via a membrane transporter or channel, followed by their rapid sequestering into PSVs via a second carrier *(26,40)* (Fig. 2), by analogy with the sequestering of recaptured neurotransmitters into synaptic vesicles by vesicular monoamine transporters of the Slc18 subfamily *(43)*.

We first demonstrated that the first step in polyamine transport clearly did not rely on clathrin-dependent internalization of a hypothetical polyamine receptor because temperature-sensitive CHO cell mutants deficient in receptor-mediated endocytosis (END1) *(44)* displayed no defect in the uptake of Spd-C_2-BODIPY *(40)*. On the other hand, Spd-C_2-BODIPY colocalized almost perfectly with LysoTracker Red, a probe that exclusively labels acidic subcellular compartments. Attempts to fix the internalized polyamine probe under conditions suitable for immunocytochemical studies did not succeed, and therefore it was not possible to identify the exact population of vesicles to which PSVs belong. To address this problem, we labeled instead two types of CHO mutant cell lines, LEX1 and LEX2, which bear defects in the pre-lysosome/late endosome fusion mechanism and in the maturation of multivesicular bodies, respectively *(45,46)*. Abnormal structures accumulating in LEX1 mutants can be readily identified as prelysosome/late endosome hybrid structures in the form of perinuclear fusiform aggregates centered around microtubule organizing centers. Likewise, LEX2 mutants accumulate larger number of multivesicular bodies than parental cells. Colocalization studies demonstrated that PSVs coincide with both types of LysoTracker Red-positive, aberrant vesicles in LEX1 and LEX2 mutants, to an even greater extent than in wild-type cells *(40)*. Therefore, one could assign PSVs to vesicles belonging to the late endocytic pathway, including multivesicular bodies, lysosomes, and late endosomes. Interestingly, Spd-C_2-BODIPY was found to accumulate to a subpopulation of the *trans*-Golgi network (TGN), which is also characterized by an acidic lumen *(40)*.

A plausible explanation for the selective accumulation of the polyamine probe into acidic vesicles might have been “amine trapping.” The latter phenomenon involves free

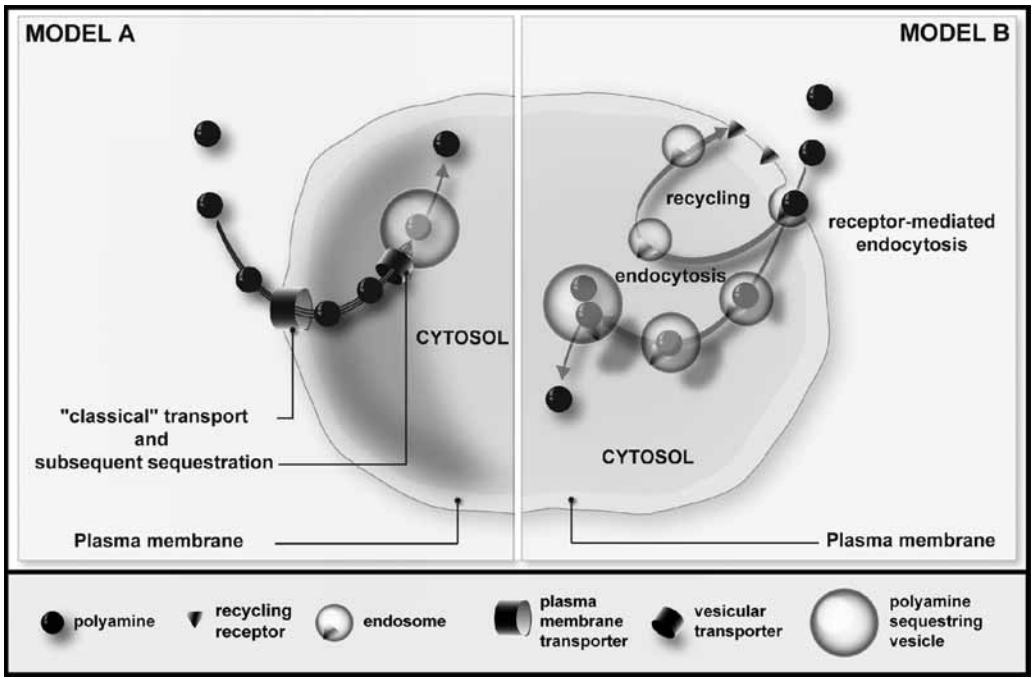

Fig. 2. Two simple models initially proposed to account for the intravesicular pattern of accumulation observed for polyamine fluoroprobes. Model A, the two-step (or two-permease) model: the polyamine is first transported via a classic permease or a channel across the plasma membrane into the cytosol, and is subsequently transported inside vesicular structures (or polyamine-sequestering vesicles, PSVs) via a second transporter; sequestered polyamines could be secondarily released from these vesicles according to cellular needs. Model B, the receptor-mediated endocytosis (or single-permease) model: the polyamine binds to a cell-surface receptor, and the polyamine–receptor complex is then internalized by endocytosis. Acidification of the endosome favors dissociation of the complex, and the free polyamine exits from the endosome (e.g., using a proton-coupled symporter).

diffusion of the uncharged form of weakly basic, amphipathic amines (e.g., LysoTracker Red) through the membrane of acidic compartments, followed by protonation of the amino group and selective retention of the cationic species inside the vesicle *(47)*. However, as with natural polyamines, Spd-C_2-BODIPY does not qualify a weakly basic compound because of the high pK_a values of its primary amino groups. Definitive evidence that PSV labeling by polyamine probes does not merely result from amine trapping came from the demonstration that polyamine transport-deficient CHO cells possess a normal complement of acidic vesicles but are completely resistant to labeling by Spd-C_2-BODIPY *(40)*.

Therefore, these colocalization studies identified a substantial percentage of PSVs as late endocytic and TGN vesicles. Such a specific distribution was consistent with the hypothesis that polyamines accumulate into PSVs via an antiporter-mediated mechanism that uses the outwardly directed proton gradient as a free energy source for transport, much as with the mechanism responsible for neurotransmitter sequestration into synaptic vesicles by vesicular monoamine transporters *(43)*. Indeed, bafilomycin A_1, a potent inhibitor of the vacuolar H^+-ATPase (V-ATPase), potently suppressed accumulation of the probe inside PSVs by more than 90%. However, a marked increase of diffuse cytoplasmic labeling was observed in bafilomycin-treated cells, indicating that V-ATPase inhibition still allows residual internalization of the probe. Conversely, when cells were preloaded with Spd-C_2-BODIPY and then incubated with bafilomycin A_1 in the absence of the probe, the fluorescent polyamine slowly emptied from PSVs over the course of more than 2 h. Initial PSV labeling at the start of chase persisted for at least 30 min, despite a complete dissipation of the proton gradient. Interestingly, the progressive decrease in intensity of PSV labeling was paralleled by a transient increase in diffuse cytoplasmic labeling *(40)*.

It is clear from the latter studies that the pattern of vesicular accumulation of fluorescent polyamines results from their active transport into multivesicular bodies, late endosomes, lysosomes, and TGN vesicles, at the expense of the proton gradient maintained by the V-ATPase (Fig. 3). Proton-pumping activity is required not only for polyamine influx into the PSVs, but also for their vesicular sequestration, indicating the existence of an inverse pathway for efflux across the PSV membrane. Moreover, enrichment in intermediate stages of accumulation as a result of V-ATPase inhibition supports the notion that the polyamine probes accumulate or efflux from PSVs directly from or onto the cytosol.

That classic, receptor-mediated endocytosis is not required for polyamine accumulation does not rule out clathrin-independent modes of endocytosis, including caveolar endocytosis (or potocytosis) *(41,48)*. The only experimental support for such an initial step of polyamine delivery to the cytoplasm has come from studies on spermine release from glypican-1 via a caveolin-dependent endocytic process *(41)*. However, putrescine and spermidine do not significantly bind heparan sulfate *(49)*, and it is thus unlikely that such a mode of spermine entry reflects a general mechanism for the polyamine transport system. Furthermore, a key role for caveolin-1 in polyamine internalization is difficult to reconcile with the known downregulation or suppression of caveolin-1 expression in many cancer types *(50)* and the elevation of polyamine uptake activity

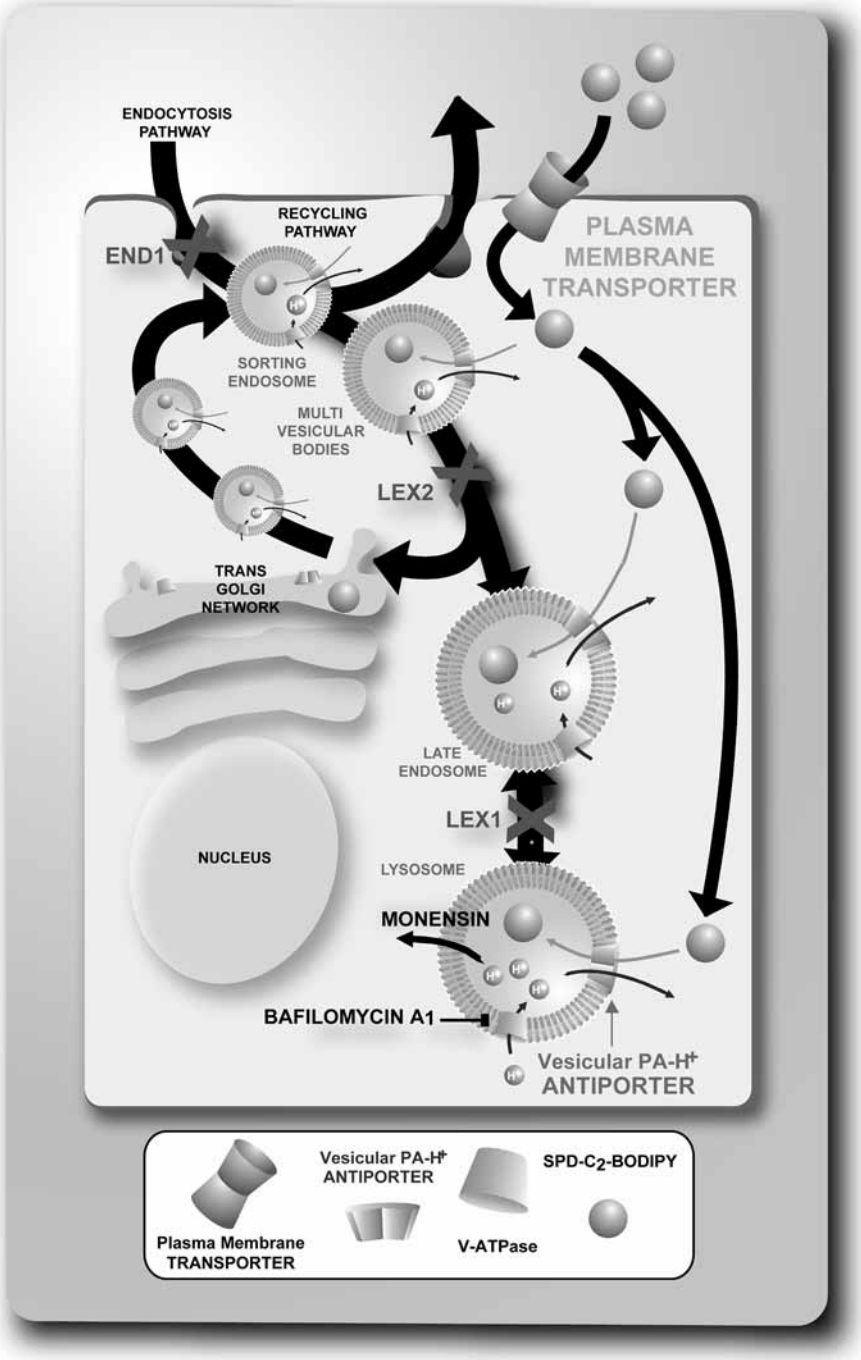
ENDOCYTOSIS PATHWAY
RECYCLING PATHWAY
END1
PLASMA MEMBRANE TRANSPORTER
SORTING ENDOSOME
MULTI VESICULAR BODIES
LEX2
TRANS GOLGI NETWORK
LATE ENDOSOME
LEX1
NUCLEUS
LYSOSOME
MONENSIN
BAFILOMYCIN A1
Vesicular PA-H+ ANTIPORTER
Vesicular PA-H+ ANTIPORTER
SPD-C2-BODIPY
Plasma Membrane TRANSPORTER
V-ATPase

Fig. 3

that occurs on cell transformation *(13,15)*. Nevertheless, an accessory contribution of heparan sulfate binding may well facilitate spermine uptake in mammalian cells, as supported by recent studies *(41)*.

4. A General Model for Polyamine Transport and Sequestration in Mammalian Cells

4.1. The Two-Step Mechanism of Polyamine Internalization

Our investigations of the mechanism of Spd-C_2-BODIPY internalization have led us to define two successive steps in the transport process: an initial influx across the plasma membrane into the cytosolic compartment via a classic permease or channel, immediately followed by its accumulation from the cytosol into PSVs via a H^+-dependent vesicular antiporter (Fig. 3) *(40)*. In this model, the equilibrium among the extracellular medium, cytosolic, and vesicular compartments strongly favors accumulation of the polyamine conjugate into PSVs, thereby reducing considerably its cytosolic concentration. Thus the steep proton gradient and the high capacity of the late endocytic compartment provide a mechanism for the rapid and efficient sequestration of the bulk of internalized Spd-C_2-BODIPY from the cytosol. Importantly, sequestering of the polyamine conjugate into PSVs is a reversible process. A pathway for the efflux of sequestered Spd-C_2-BODIPY exists, although it is as yet unclear whether it is mediated by the reverse mode of operation of the putative polyamine/proton antiporters or a parallel transport agency.

A most important feature of this model is that the steadystate sequestering of polyamines from the cytosol into PSVs creates a driving force for polyamine uptake by favoring influx at the plasma membrane level. Although no quantitative measurement of the probe concentration in the cytosol was attempted, the very low level of Spd-C_2-BODIPY detected in the cytosol at any time indicates that the constant uptake into PSVs obviously maintains an inwardly directed diffusion gradient across the plasma membrane. In fact, the quantitative importance of the intravesicular accumulation step in the cellular capacity for transport of the probe is clearly illustrated by the dramatic reduction of total Spd-C_2-BODIPY uptake observed on the inhibition of V-ATPase

Fig. 3. Model proposed and evidence for the two-step mechanism of polyamine accumulation in mammalian cells. Polyamines (PA) (here represented as the Spd-C_2-BODIPY probe) are first transported from the extracellular space into the cytosol via a classic plasma membrane permease (or channel). A second permease functioning as a PA/H^+ exchanger uses the proton gradient as a driving force (highly sensitive to bafilomycin A_1 and monensin) to internalize polyamines inside polyamine-sequestering vesicles (PSVs). PSVs include lysosomes and late endosomes, as demonstrated using LEX1 and LEX2 mutants, but do not include early endosomes as shown using END1 mutants. A subset of the *trans*-Golgi network can also accumulate polyamines, which might be explained by the known pathways connecting the Golgi and the late endocytic compartment. A limited fraction of recycling endosomes also behave as PSVs, suggesting that vesicular polyamine transporters are initially inserted in the sorting endosome. The exit pathway for efflux of polyamines sequestered in the PSVs is not illustrated here for the sake of simplicity. (For other details, *see* main text and ref. *40*.)

activity. Thus transport into PSVs is the main limiting step in total Spd-C_2-BODIPY transport. As a major corollary of this observation, the plasma membrane step of the transport process may not require, in principle, the presence of an active transporter or an ion-coupled carrier, but might be carried out by a facilitator or a channel. Indeed, the ability of polyamines to permeate some ion channels has already been recognized *(51)*. Moreover, the strong dependence of polyamine transport on an electronegative membrane potential *(20,21)* is consistent with the behavior of a plasma membrane channel. On the other hand, the wide spectrum of substrates accommodated by the mammalian polyamine transport system *(15,16,24,27)* may be hard to reconcile with a channel-mediated process, as shown by the inability of large polyamine conjugates to permeate ion channels *(51)*.

4.2. Implications of the Two-Step Model for Polyamine Transport and Homeostasis

The real value of any model is measured by how well it fits to the body of experimental evidence, and by its intrinsic ability to implicitly predict properties of the system. Because vesicular accumulation is a property shared by all fluorescent polyamine conjugates studied to date *(25,26,39,40)*, including spermidine and spermine derivatives with substituents borne on either imino or terminal amino groups, the nature and role of the late endocytic compartment, as well as the mechanism governing the uptake and sequestration of Spd-C_2-BODIPY are likely to be relevant to the homeostasis of natural polyamines. The biochemical properties and behavior of *N*-{spermidine-[N^4-(3-aminopropyl)]}anthranylamide (Fig. 1B) and Spd-C_2-BODIPY closely mimic those of natural polyamines, including the upregulation of their transport on prior depletion of the polyamine pool and the feedback inhibition of their intracellular influx *(25,26)*. Thus the general organization of transport as a two-step process, and the prevalence of an extensive pool of intravesicular molecules, are properties likely apply to natural polyamines.

The model proposed in Fig. 3 has major implications for our understanding of the regulation of polyamine transport and on polyamine compartmentalization and homeostasis. First, this model predicts the existence of at least two general classes of polyamine transporters that coordinately control the accumulation of polyamines at the cytosolic and vesicular levels. Although the identity of the plasma membrane carrier still remains elusive, we now possess strong arguments pointing to the existence of proton-dependent antiporters located in late endosomes and lysosomes and that use polyamines as major substrates. It is noteworthy that a H^+/organic amine exchange activity has been biochemically detected in rat liver lysosomes, using triethylammonium as substrate *(52)*. Future investigations should determine whether the latter activity is related to polyamine transport into PSVs. The heretofore unrecognized notion of vesicular polyamine carriers should contribute to clarify the very complex physiological behavior of polyamine transport in mammalian cells. The future identification and characterization of each class of polyamine transporters at the molecular level will likely help to better delineate the respective properties of each transporter population.

Second, the proposed paradigm for polyamine compartmentalization inside mammalian cells implicitly suggests a novel mechanism for the regulation of polyamine

homeostasis by its main effectors, namely the AZ. The molecular nature of the mechanism governing the inhibitory action of AZ on polyamine uptake remains unknown. Nevertheless, it is clear that AZ induction must result from increases in the concentration of polyamines prevailing in the local ribosome environment *(1)*. The current model predicts that cytosolic polyamine levels would be narrowly dependent on vesicular antiport activity, which, in turn, is at least partly dependent on the transmembrane polyamine and proton electrochemical potentials. Therefore, as polyamine concentrations inside PSVs increase, the rate of intravesicular uptake would decrease, resulting in a rapid increase in cytosolic levels and induction of AZ. Such a mechanism would therefore predict that the total capacity of PSVs and the expression level of the vesicular polyamine transporters are the main factors governing the induction of AZ. Conversely, depletion of the polyamine pool such as that observed on treatment with DFMO, which translates mainly into a decrease in intravesicular polyamine concentrations in this model, will be readily reflected as an enhanced rate of vesicular polyamine/proton antiport, and thus, total rate of polyamine accumulation.

Evidently, the suppression of net polyamine uptake exerted by AZ could theoretically involve an interaction of AZ with either plasma membrane or vesicular transporters, or both classes of carriers. Interestingly, preliminary observations using Spd-C_2-BODIPY suggest that cytosolic levels of the fluoroprobe dramatically increase under conditions whereby AZ induction is prevented (Gagnon, B., Soulet, D., Rivest, S., Audette, M. and Poulin, R., unpublished results). These data suggest that inhibition by AZ may result mainly or exclusively from their interaction with the plasma membrane polyamine carrier.

4.3. PSVs as Physiologically Regulated Stores for Intracellular Polyamines

The existence of PSVs as a major reservoir for intracellular polyamines immediately reminds key aspects of the organization of Ca^{2+} homeostasis. Indeed, the role of PSVs may be analogous to that of the endoplasmic/sarcoplasmic reticulum in the case of Ca^{2+} homeostasis *(33)* in providing a readily accessible reservoir for cations exerting pleiotropic actions in the cell. Such an organization for the regulation of the "free" polyamine pool offers several conceptual advantages over the previously proposed hypothesis of polyamine sequestration by noncovalent binding to macromolecules (mainly ribosomal RNA and ATP) *(5)*. The latter hypothesis implicitly requires physicochemical shifts in the equilibrium between polyamines and the number of binding sites as a chief mechanism leading to fluctuations in the size of the free polyamine pool. Whereas contributions of the latter mechanism to adjusting cytosolic polyamine concentrations cannot be discarded, mobilization of polyamines from PSVs would provide much more flexibility, reversibility, and velocity of changes than passive changes in binding equilibrium.

By analogy with Ca^{2+} stores, one of the main functions of PSVs might be the removal of the biologically active polyamines from the cytosol to allow differential responses to various stimuli. Polyamines exert a plethora of physiological actions in the cell, and the question of the dynamic, active coordination of these actions has long

been a very challenging one in the study of polyamines. The existence of PSVs brings a new perspective to the problem of the segregation of polyamines from the main cytosolic compartment. For instance, polyamines have been ascribed both pro- and antiapoptotic roles *(32)*, and the storage of these compounds in a form that can be rapidly mobilized under certain conditions may provide a physical basis for understanding these apparently contradictory properties.

A hypothetical role of PSVs in the rapid mobilization of sequestered polyamines into the cytosol would provide a physiological basis for the mediation of various actions proposed for polyamines, such as the transduction of specific signals *(53)* and the gating of ion channels *(54)*. A general acceptance of such physiological roles postulated for polyamines has been hampered by the lack of intracellular pathways allowing the rapid adjustment of cytosolic concentrations. For instance, changes in the inward rectification of gating for some ion channels requires fluctuations in cytosolic polyamine concentrations in the submicromolar range *(54)*, a condition that could be readily fulfilled by the controlled release of polyamines from the PSV pool. Thus the notion of vesicular polyamine pools that quickly and reversibly limit the interaction of polyamines with their multiple intracellular sites of action would provide the pathways with fast kinetics that were previously lacking in our general picture of polyamine homeostasis. Further investigations on the mechanism regulating the size of the polyamine pools inside PSVs should provide foundations for the future assessment of such a hypothesis.

4.4. PSVs, Synaptic Vesicles, and Vacuoles

As mentioned in Subheading 3.2., it comes to mind that the two-step model of polyamine internalization bears an overall similarity to the mode of neurotransmitter recapture by neuronal and neuroendocrine cells. Indeed, acetylcholine, monoamine neurotransmitters (e.g., epinephrine, dopamine, histamine), and amino acids neurotransmitters (e.g., glutamate, glycine) are accumulated from the extracellular space via a conceptually similar pattern of sequential transport first across the plasma membrane, followed by the vesicular membrane *(55)*. Transport across the plasma membrane for these substrates is carried out by various permease types, whereas accumulation into synaptic vesicles or analogous secretory vesicles is mediated by H^+-coupled antiporters belonging to three different permease groups *(43)*. Despite the structural diversity between the different permeases used for the transport of these ligands, it is remarkable that a relatively uniform pattern has been preserved for the storage of molecules compartmentalized in secretory vesicles. Thus the general organization of the two-step, two-compartment model for polyamine transport proposed here is by no means unprecedented. Interestingly, a biochemical activity responsible for the H^+-dependent accumulation of polyamines within synaptic vesicles has recently been described *(3)*, reinforcing the notion that vesicular polyamine transporters do exist in mammalian cells.

Storage of polyamines inside vesicular structures at the expense of the proton gradient has its obvious counterpart in plants and fungi in the form of the vacuole. Although our knowledge of polyamine transport and compartmentalization in vacuoles is only emerging from studies in the yeast *Saccharomyces cerevisiae* (*see* Chapter 25), PSVs

may share the same evolutionary and physiological function as the yeast vacuole, in as much as they are basically identical to the late endosomal and lysosomal compartments. The possibility that PSVs also include endosomes targeted to the secretory pathway and exocytosis is by no means excluded, a possibility consistent with the mechanistic homology between PSVs and synaptic vesicles in the vesicular amine storage process.

5. Conclusion

The recent realization that the late endocytic compartment is likely the main storage compartment of polyamines in some mammalian cells opens exciting avenues of research to better understand the function and regulation of vesicular polyamine transport, its more general distribution in various tissues, and how it is integrated with polyamine metabolism and with the numerous functions of these molecules. The existence of PSVs adds new levels of complexity to the problem of polyamine transport in mammalian cells. Moreover, the almost exclusive presence of PSVs in the cytoplasm in cell types thus far examined challenges a widely held notion that the nucleus is the main site of polyamine compartmentalization in vertebrate cells *(35)*. More detailed studies of the behavior of these structures throughout the cell cycle might help to clarify this important question.

Acknowledgments

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References

1. Coffino, P. (2001) Regulation of cellular polyamines by antizyme. *Nat. Rev. Mol. Cell. Biol.* **2,** 188–194.
2. Toninello, A., Salvi, M., and Mondovi, B. (2004) Interaction of biologically active amines with mitochondria and their role in the mitochondrial-mediated pathway of apoptosis. *Curr. Med. Chem.* **11,** 2349–2374.
3. Masuko, T., Kusama-Eguchi, K., Sakata, K., et al. (2003) Polyamine transport, accumulation, and release in brain. *J. Neurochem.* **84,** 610–617.
4. Murai, N., Murakami, Y., and Matsufuji, S. (2003) Identification of nuclear export signals in antizyme-1. *J. Biol. Chem.* **278,** 44,791–44,798.
5. Watanabe, S., Kusama-Eguchi, K., Kobayashi, H., and Igarashi, K. (1991) Estimation of polyamine binding to macromolecules and ATP in bovine lymphocytes and rat liver. *J. Biol. Chem.* **266,** 20,803–20,809.
6. Kanamoto, R., Kameji, T., Iwashita, S., Igarashi, K., and Hayashi, S. (1993) Spermidine-induced destabilization of ornithine decarboxylase (ODC) is mediated by accumulation of antizyme in ODC-overproducing variant cells. *J. Biol. Chem.* **268,** 9393–9399.
7. Ignatenko, N. A. and Gerner, E. W. (1996) Growth arrest- and polyamine-dependent expression of spermidine/spermine N^1-acetyltransferase in human tumor cells. *Cell Growth Differ.* **7,** 481–486.
8. Erwin, B. G. and Pegg, A. E. (1986) Regulation of spermidine/spermine N^1-acetyltransferase in L6 cells by polyamines and related compounds. *Biochem. J.* **238,** 581–587.
9. Howard, M. T., Shirts, B. H., Zhou, J., et al. (2001) Cell culture analysis of the regulatory frameshift event required for the expression of mammalian antizymes. *Genes Cells* **6,** 931–941.

10. Mitchell, J. L., Leyser, A., Holtorff, M. S., et al. (2002) Antizyme induction by polyamine analogues as a factor of cell growth inhibition. *Biochem. J.* **366,** 663–671.
11. Fogel-Petrovic, M., Vujcic, S., Brown, P. J., Haddox, M. K., and Porter, C. W. (1996) Effects of polyamines, polyamine analogs, and inhibitors of protein synthesis on spermidine-spermine N^1-acetyltransferase gene expression. *Biochemistry* **35,** 14,436–14,444.
12. Hasne, M. P. and Ullman, B. (2005) Identification and characterization of a polyamine permease from the protozoan parasite *Leishmania major*. *J. Biol. Chem.* **280,** 15,188–15,194.
13. Seiler, N., Delcros, J. G., and Moulinoux, J. P. (1996) Polyamine transport in mammalian cells. An update. *Int. J. Biochem. Cell. Biol.* **28,** 843–861.
14. Poulin, R., Kaouass, M., Soulet, D., Covassin, L., Robert, E., and Audette, M. (2002) Cellular permeability to polyamines. In: *COST 917—Biogenically Active Amines in Food, vol. V* (Morgan, D. L. M., Hirvi, T., Dandrifosse, G., Deloyer, P., and White, A., eds.), European Community Publications, Luxembourg City, Luxembourg, pp. 3–21.
15. Seiler, N. (2003) Thirty years of polyamine-related approaches to cancer therapy. Retrospect and prospect. Part 2. Structural analogues and derivatives. *Curr. Drug Targets* **4,** 565–585.
16. Lessard, M., Zhao, C., Singh, S. M., and Poulin, R. (1995) Hormonal and feedback regulation of putrescine and spermidine transport in human breast cancer cells. *J. Biol. Chem.* **270,** 1685–1694.
17. Byers, T. L., Kameji, R., Rannels, D. E., and Pegg, A. E. (1987) Multiple pathways for uptake of paraquat, methylglyoxal bis(guanylhydrazone), and polyamines. *Am. J. Physiol.* **252,** C663–C669.
18. Ruchko, M., Gillespie, M. N., Weeks, R. S., Olson, J W., and Babal, P. (2003) Putrescine transport in hypoxic rat main PASMCs is required for p38 MAP kinase activation. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **284,** L179–L186.
19. Nicolet, T. G., Scemama, J. L., Pradayrol, L., Seva, C., and Vaysse, N. (1990) Characterization of putrescine- and spermidine-transport systems of a rat pancreatic acinar tumoral cell line (AR4–2J). *Biochem. J.* **269,** 629–632.
20. Poulin, R., Zhao, C., Verma, S., Charest-Gaudreault, R., and Audette, M. (1998) Dependence of mammalian putrescine and spermidine transport on plasma-membrane potential: Identification of an amiloride binding site on the putrescine carrier. *Biochem. J.* **330,** 1283–1291.
21. Poulin, R., Lessard, M., and Zhao, C. (1995) Inorganic cation dependence of putrescine and spermidine transport in human breast cancer cells. *J. Biol. Chem.* **270,** 1695–1704.
22. Pohjanpelto, P. (1976) Putrescine transport is greatly increased in human fibroblasts initiated to proliferate. *J. Cell. Biol.* **68,** 512–520.
23. Torossian, K., Audette, M., and Poulin, R. (1996) Substrate protection against inactivation of the mammalian polyamine- transport system by 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide. *Biochem. J.* **319,** 21–26.
24. Li, Y., MacKerell, A. D., Jr., Egorin, M. J., et al. (1997) Comparative molecular field analysis-based predictive model of structure-function relationships of polyamine transport inhibitors in L1210 cells. *Cancer Res.* **57,** 234–239.
25. Cullis, P. M., Green, R. E., Merson-Davies, L., and Travis, N. (1999) Probing the mechanism of transport and compartmentalisation of polyamines in mammalian cells. *Chem. Biol.* **6,** 717–729.
26. Soulet, D., Covassin, L., Kaouass, M., Charest-Gaudreault, R., Audette, M., and Poulin, R. (2002) Role of endocytosis in the internalization of spermidine-C_2-BODIPY, a highly fluorescent probe of polyamine transport. *Biochem. J.* **367,** 347–357.

27. Wang, C., Delcros, J. G., Biggerstaff, J., and Phanstiel, O.T. (2003) Molecular requirements for targeting the polyamine transport system. Synthesis and biological evaluation of polyamine-anthracene conjugates. *J. Med. Chem.* **46,** 2672–2682.
28. Ambudkar, S. V., Kimchi-Sarfaty, C., Sauna, Z. E., and Gottesman, M. M. (2003) P-glycoprotein: From genomics to mechanism. *Oncogene* **22,** 7468–7485.
29. Aziz, S. M., Yatin, M., Worthen, D. R., Lipke, D. W., and Crooks, P. A. (1998) A novel technique for visualizing the intracellular localization and distribution of transported polyamines in cultured pulmonary artery smooth muscle cells. *J. Pharm. Biomed. Anal.* **17,** 307–320.
30. Newman, R. M., Mobascher, A., Mangold, U., et al. (2004) Antizyme targets cyclin D1 for degradation. A novel mechanism for cell growth repression. *J. Biol. Chem.* **279,** 41,504–41,511.
31. Cohen, S. S. (1998) *A Guide to the Polyamines.* Oxford University Press, New York, NY.
32. Schipper, R. G., Penning, L. C., and Verhofstad, A. A. (2000) Involvement of polyamines in apoptosis. Facts and controversies: effectors or protectors? *Semin. Cancer Biol.* **10,** 55–68.
33. Venkatachalam, K., van Rossum, D. B., Patterson, R. L., Ma, H. T., and Gill, D. L. (2002) The cellular and molecular basis of store-operated calcium entry. *Nat. Cell. Biol.* **4,** E263–E272.
34. Hougaard, D. M. (1992) Polyamine cytochemistry: Localization and possible functions of polyamines. *Int. Rev. Cytol.* **138,** 51–88.
35. Roch, A. M., Nicolas, M. T., and Quash, G. (1997) Ultrastructural immunolocalization of polyamines in HeLa cells subjected to fast-freezing fixation and freeze substitution. *Histochem. Cell. Biol.* **107,** 303–312.
36. Fujiwara, K., Bai, G., and Kitagawa, T. (1997) Polyamine-like immunoreactivity in rat neurons. *Brain Res.* **767,** 166–171.
37. Delcros, J.-G., Loeuillet, L., Chamaillard, L., et al. (1997) Flow cytometric analysis of in vivo polyamine deprivation in Lewis lung carcinoma (3LL) cells using the monoclonal antibody SPM8-2. *Cytometry* **27,** 255–261.
38. Schipper, R. G., Cuijpers, V. M., De Groot, L. H., Thio, M., and Verhofstad, A. A. (2004) Intracellular localization of ornithine decarboxylase and its regulatory protein, antizyme-1. *J. Histochem. Cytochem.* **52,** 1259–1266.
39. Wang, C., Delcros, J. G., Biggerstaff, J., and Phanstiel, O. T. (2003) Synthesis and biological evaluation of N^1-(anthracen-9-ylmethyl)triamines as molecular recognition elements for the polyamine transporter. *J. Med. Chem.* **46,** 2663–2671.
40. Soulet, D., Gagnon, B., Rivest, S., Audette, M., and Poulin, R. (2004) A fluorescent probe of polyamine transport accumulates into intracellular acidic vesicles via a two-step mechanism. *J. Biol. Chem.* **279,** 49,355–49,366.
41. Belting, M., Mani, K., Jönsson, M., et al. (2003) Glypican-1 is a vehicle for polyamine uptake in mammalian cells. A pivotal role for nitrosothiol-derived nitric oxide. *J. Biol. Chem.* **278,** 47,181–47,189.
42. Gruenheid, S., Canonne-Hergaux, F., Gauthier, S., Hackam, D. J., Grinstein, S., and Gros, P. (1999) The iron transport protein NRAMP2 is an integral membrane glycoprotein that colocalizes with transferrin in recycling endosomes. *J. Exp. Med.* **189,** 831–841.
43. Gasnier, B. (2000) The loading of neurotransmitters into synaptic vesicles. *Biochimie* **82,** 327–337.
44. Roff, C. F., Fuchs, R., Mellman, I., and Robbins, A. R. (1986) Chinese hamster ovary cell mutants with temperature-sensitive defects in endocytosis. I. Loss of function on shifting to the nonpermissive temperature. *J. Cell. Biol.* **103,** 2283–2297.

45. Ohashi, M., Miwako, I., Nakamura, K., et al. (1999) An arrested late endosome-lysosome intermediate aggregate observed in a Chinese hamster ovary cell mutant isolated by novel three-step screening. *J. Cell. Sci.* **112,** 1125–1138.
46. Ohashi, M., Miwako, I., Yamamoto, A., and Nagayama, K. (2000) Arrested maturing multivesicular endosomes observed in a Chinese hamster ovary cell mutant, LEX2, isolated by repeated flow-cytometric cell sorting. *J. Cell. Sci.* **113,** 2187–2205.
47. de Duve, C., de Barsy, T., Poole, B., Trouet, A., Tulkens, P., and Van Hoof, F. (1974) Commentary. Lysosomotropic agents. *Biochem. Pharmacol.* **23,** 2495–2531.
48. Nichols, B. (2003) Caveosomes and endocytosis of lipid rafts. *J. Cell. Sci.* **116,** 4707–4714.
49. Belting, M., Havsmark, B., Jönsson, M., Persson, S., and Fransson, L. Å. (1996) Heparan sulphate/heparin glycosaminoglycans with strong affinity for the growth-promoter spermine have high antiproliferative activity. *Glycobiology* **6,** 121–129.
50. Cohen, A. W., Hnasko, R., Schubert, W., Lisanti, M. P. (2004) Role of caveolae and caveolins in health and disease. *Physiol. Rev.* **84,** 1341–1379.
51. Kerschbaum, H. H., Kozak, J. A., and Cahalan, M. D. (2003) Polyvalent cations as permeant probes of MIC and TRPM7 pores. *Biophys. J.* **84,** 2293–2305.
52. Moseley, R. H., and Van Dyke, R. W. (1995) Organic cation transport by rat liver lysosomes. *Am. J. Physiol.* **268,** G480–G486.
53. Paasinen-Sohns, A., Kielosto, M., Kääriäinen, E., et al. (2000) c-Jun activation-dependent tumorigenic transformation induced paradoxically by overexpression or block of *S*-adenosylmethionine decarboxylase. *J. Cell. Biol.* **151,** 801–810.
54. Williams, K. (1997) Interactions of polyamines with ion channels. *Biochem. J.* **325,** 289–297.
55. Liu, Y. and Edwards, R. H. (1997) The role of vesicular transport proteins in synaptic transmission and neural degeneration. *Annu. Rev. Neurosci.* **20,** 125–156.

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Bacterial and Eukaryotic Transport Systems

Kazuei Igarashi and Keiko Kashiwagi

1. Introduction

Polyamines (putrescine, spermidine, and spermine) are essential for normal cell growth *(1,2)*, and their contents in cells are maintained by biosynthesis, degradation, and transport of polyamines *(3–6)*. They are among the major polycations in cells, together with Ca^{2+} and Mg^{2+}. Polyamines and Mg^{2+}, which are present in higher concentrations than Ca^{2+} in cytoplasm and nuclei, can bind to intracellular polyanions, such as nucleic acids and adenosine triphosphate (ATP), to modulate their functions *(7,8)*. Polyamines also can bind to specific proteins, such as *N*-methyl-D-aspartate receptors and some kinds of K^+ channels, to regulate their activities *(9)*.

As for polyamine transport, several transporters were identified in *Escherichia coli* and yeast, but no polyamine transporters have thus far been identified in mammalian cells. Recently, it has been reported that glypican-1 is a vehicle for polyamine uptake in mammalian cells *(10)*. In this chapter, we will describe the properties of polyamine transport in *E. coli*, yeast, and mammalian cells.

2. Polyamine Transport in *E. coli*

E. coli contain putrescine and spermidine. In addition, cadaverine and aminopropylcadaverine can function similarly to putrescine and spermidine *(11)*. Therefore, we first tried to identify the genes encoding the transport proteins for putrescine, spermidine, and cadaverine. To identify these genes, a mutant that is deficient in polyamine transport was first isolated. Genes for polyamine transport systems were then isolated by transforming the mutant with DNA fragments using pACYC184 as a vector. One clone for the genes encoding proteins catalyzing both putrescine and spermidine uptake (pPT104) was isolated. Two clones for the genes of proteins catalyzing only putrescine uptake (pPT79 and pPT71) were obtained *(12)*. The *Km* values for spermidine and putrescine of the polyamine transport system encoded by pPT104 were 0.1 and 1.5 μ*M*, respectively. The *Km* value for putrescine of the putrescine transport systems

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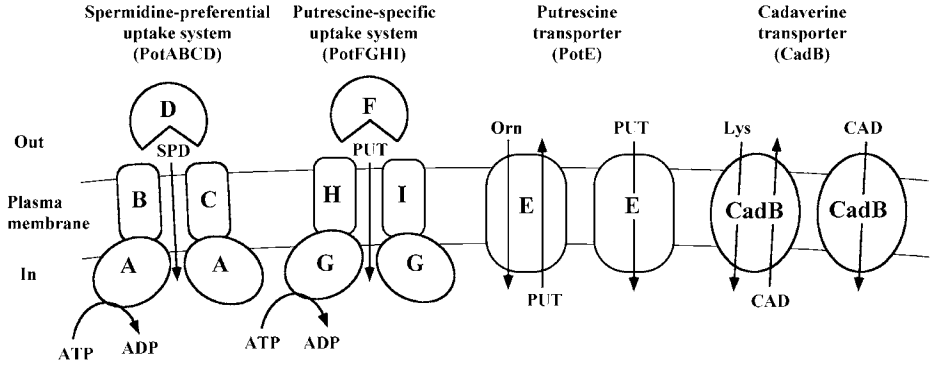


Fig. 1. Polyamine transport systems in *Escherichia coli*.

encoded by pPT79 and pPT71 was 0.5 and 1.8 μ*M*, respectively. Subsequently, *cadB* encoding the cadaverine transporter was identified *(13,14)*.

Polyamine transporters are classified into two groups: one consists of ABC (ATP-binding cassette) transporters encoded by pPT104 and pPT79 and the other, encoded by pPT71 and *cadB*, is a protein containing 12 transmembrane segments linked by hydrophilic segments of variable length with the NH_2- and COOH-termini located in the cytoplasm (Fig. 1). Polyamine transporters encoded by pPT104 (spermidine-preferential uptake system) and by pPT79 (putrescine-specific uptake system) are ABC transporters. The spermidine preferential uptake system consists of four proteins: PotA (an ATPase), PotB and PotC (channel-forming proteins), and PotD (a substrate-binding protein) *(15)*. Similarly, the putrescine-uptake system also consists of four proteins: PotF (a substrate-binding protein), PotG (an ATPase), and PotH and PotI (channel-forming proteins) *(16)*.

To determine whether all four proteins are necessary for spermidine uptake in the PotA/B/C/D system, the genes for each protein were individually disrupted by inserting the gene for kanamycin resistance in the Pot protein gene. Spermidine uptake was not observed in *E. coli* in which any one of the four proteins was disrupted. Transformation with a plasmid containing an intact gene corresponding to the disrupted one restored spermidine-uptake activity. Thus all four proteins (PotA, PotB, PotC, and PotD) are necessary for spermidine uptake. The calculated molecular masses of PotA, PotB, PotC, and PotD were 43, 31, 29, and 39 kDa, respectively *(15)*. Based on hydrophobicity analysis, PotB and PotC contain six putative transmembrane segments linked by hydrophilic segments of variable length. PotA and PotD do not contain notable hydrophobic segments. When the amino acid sequence of PotA was compared with that of other proteins, a consensus nucleotide-binding sequence was found in PotA similar to that seen in the α and β subunits of *E. coli* ATPase *(17)*, HisP and MalK proteins *(18)*. Both HisP and MalK are membrane-associated ATPases of the histidine and maltose transport systems. We therefore speculated that PotA may be also membrane associated, and, indeed, it was shown that PotA exists mainly in the inner membrane fraction. PotA was associated with membranes through the interaction with

PotB and PotC *(19)*. Because PotA was suggested to be involved in an energy-coupling step, like HisP and MalK *(18)*, the ATP dependency of spermidine uptake was examined. ATP was found to be essential for spermidine uptake *(20)*. The ATPase activity of PotA was studied using purified PotA and a PotABC complex on inside-out membrane vesicles. It was found that PotA can form a dimer by disulfide crosslinking, but that each PotA molecule functions independently *(21)*. This is in accordance with previous results with HisP *(22,23)*, although positive cooperativity for ATP during ATP hydrolysis was reported in HisP *(24)*. When PotA was associated with the membrane proteins PotB and PotC, the *Km* value for ATP increased from 0.39 to 1.49 m*M*, and PotA became much more sensitive to inhibition by spermidine. The *Ki* value for spermidine was approx 10 μ*M* at the Pot ABC complex, and spermidine uncompetitively inhibited PotA activity, suggesting that spermidine binds at a site on PotA different from the ATP-recognizing site. The results suggest that spermidine functions as a feedback inhibitor of spermidine uptake through inhibition of the ATPase activity of PotA. Amino acid residues involved in the ATPase activity were then identified (Fig. 2) and corresponded to Cys^{54}, Val^{135}, and Asp^{172}. These results indicate that the amino acid residues necessary for ATP hydrolysis of PotA are located both within and between the two consensus amino acid sequences for nucleotide binding (GPSG$\underline{C}^{54}$GKT and LLLL$\underline{D}^{172}$E). Because the homology of amino acid sequences between PotA and HisP is relatively high, the important amino acid residues in PotA were tentatively placed on the HisP structure determined by X-ray crystal analysis *(25)*. Although Cys^{54} and Asp^{172} are located at the ATP-binding domain, the position of Val^{135} is distant from the ATP-binding domain, suggesting that Val^{135} may be important for the structure of the active site of ATP hydrolysis. HisP (258 amino acid residues) is smaller than PotA (378 amino acid residues) and MalK (372 amino acid residues). It has been reported that the COOH terminus of MalK is critical for negative regulation of the *mal* operon *(26)*, and that a mutant (E306K) of the COOH terminus of MalK affects its ATPase activity, suggesting a role for this region in the ATPase activity *(27)*. Similarly, a mutant (E297K) of the COOH terminus of PotA and the COOH-terminal truncated mutants C1 (301 amino acid residues) and C2 (239 amino acid residues) affect its ATPase activity and the spermidine inhibition of ATPase activity *(21)*. Thus there are two domains in PotA like MalK. The NH_2-terminal domain (residues 1–250) contains the ATP-binding pocket formed in part by residues Cys^{26} (binding site of 8-azide-ATP), Phe^{27}, Phe^{45}, Cys^{54}, Leu^{60}, and Leu^{76}, and the active center of the ATPase that includes Val^{135} and Asp^{172}. The COOH-terminal domain (residues 251–378) of PotA contains a site that regulates ATPase activity and a site involved in the spermidine inhibition of ATPase activity.

PotD was purified to homogeneity, and the NH_2-terminal sequence was determined by Edman degradation. The processing site of PotD by signal peptidase was between Ala^{23} and Asp^{24}. Dissociation constants of spermidine and putrescine for purified PotD under the condition of 1 m*M* Mg^{2+} and 100 m*M* K^+ at pH 7.5 were 3.2 and 100 μ*M*, respectively. These values reflect the uptake system in intact cells. There was a single binding site for spermidine or putrescine on PotD, and spermidine uptake was shown to be PotD-dependent using right-side-out membrane vesicles. The PotD protein, as with

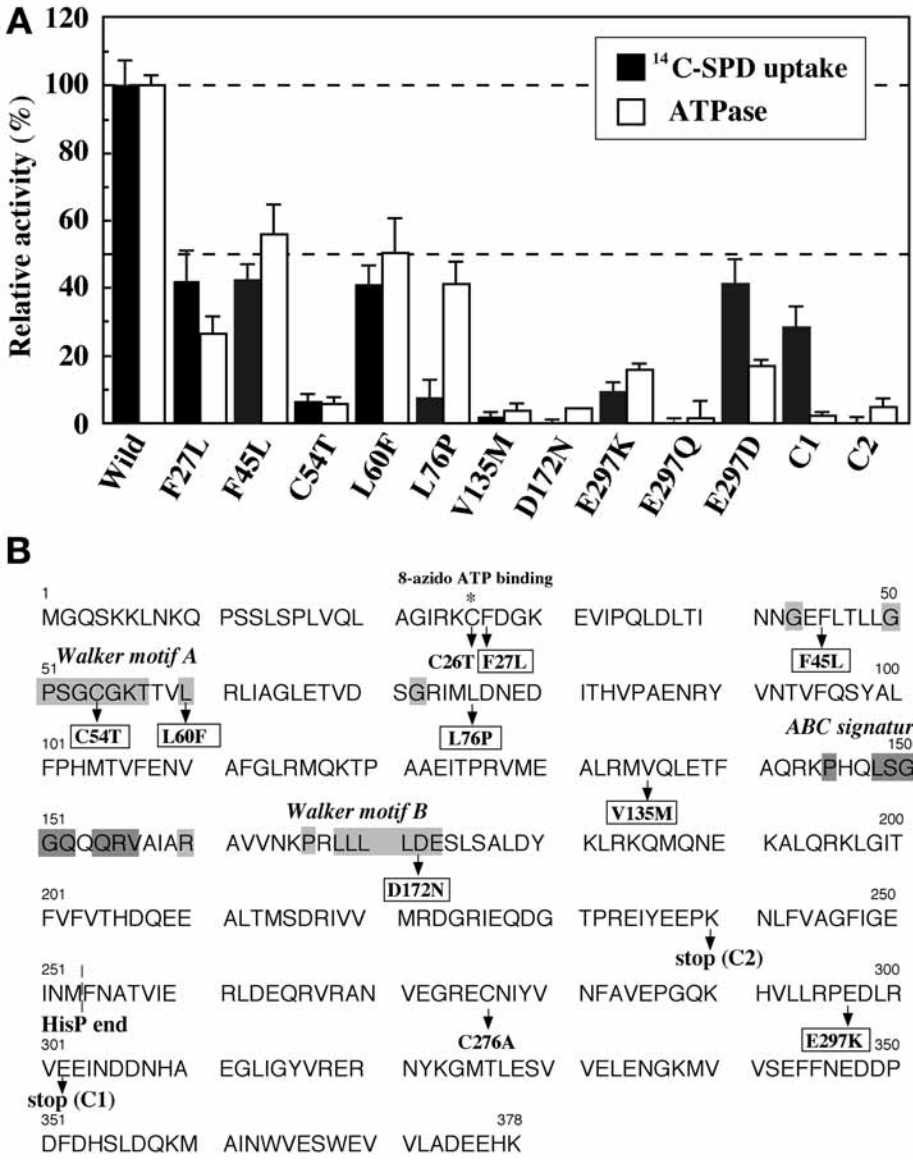


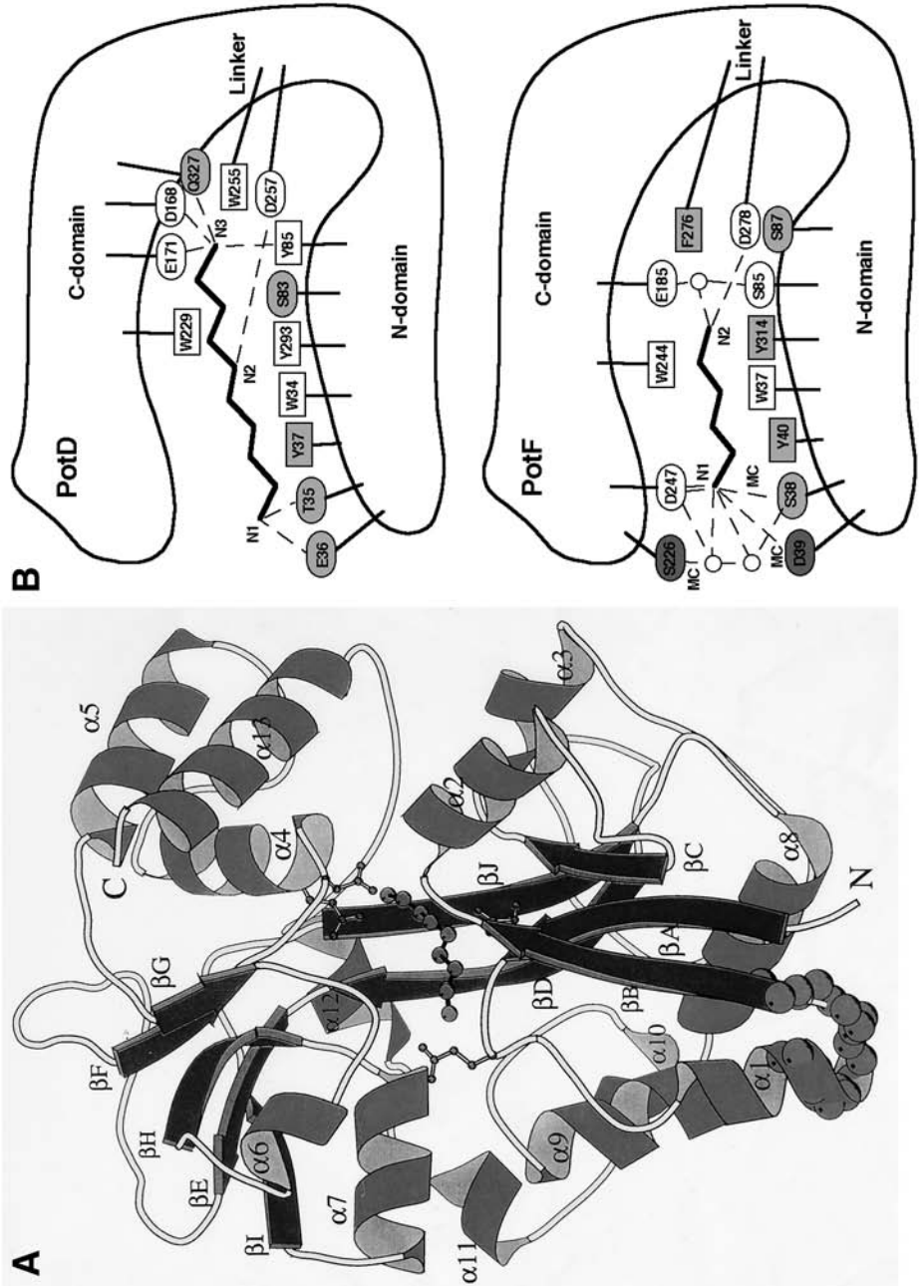
Fig. 2. Spermidine uptake and ATPase activities of various PotA mutants **(A)** and the position of mutations in the PotA mutants **(B)**. (Reproduced with permission from ref. *21*.)

other periplasmic binding proteins, consists of two domains with alternating β-α-β topology *(28)* with the polyamine-binding site located in a central cleft between these domains. Four acidic residues recognize the three positively charged nitrogen atoms of spermidine, and five aromatic side chains anchor the methylene backbone by van der

Waals interactions (Fig. 3). The overall fold of PotD is similar to that of other periplasmic-binding proteins, in particular to the maltodextrin-binding protein from *E. coli (29)*, even though the sequence identity is low. The crystal structure in the absence of spermidine is not known. However, a comparison of the PotD–spermidine structure with that of the maltodextrin-binding protein, determined in the presence and absence of its substrate, suggests that the binding of spermidine rearranges the relative orientation of the PotD domains to create a more compact structure. It was found that 13 amino acid residues were involved in binding of spermidine (Fig. 3). Among these residues, Glu^{171}, Trp^{255}, and Asp^{257} were the most important for binding of spermidine, Trp^{34}, Tyr^{85}, Asp^{168}, Trp^{229}, and Tyr^{293} had moderate contributions and the other five amino acids made a weak contribution to spermidine binding *(30)*. The dissociation constants of spermidine for PotD mutated at Glu^{171}, Trp^{255}, and Asp^{257} increased greatly compared with the other mutants. Because these three residues interact with the diaminopropane moiety of spermidine, the results agree with the finding that PotD has a higher affinity for spermidine than for putrescine. Similarly, using PotD mutants, putrescine was found to bind at the position of the diaminobutane moiety of spermidine.

Properties of the putrescine binding protein (PotF) in the putrescine-specific uptake system was also clarified by the crystal structure together with studies of mutated PotF proteins *(31)*. The structure of PotF is reminiscent of other periplasmic substrate binding proteins, with the highest structural similarity to that of PotD. Putrescine was tightly bound in a deep cleft between the two domains of PotF. The structure revealed the residues crucial for putrescine binding (Trp^{37}, Ser^{85}, Trp^{244}, Asp^{247}, and Asp^{278}) and the importance of water molecules for putrescine recognition. Two residues in PotD, Thr^{35} and Glu^{36}, the side chains of which make hydrogen bonds with the N1 nitrogen of spermidine, are replaced by Ser^{38} and Asp^{39} in PotF. In contrast to PotD, putrescine makes hydrogen bonds with the main chain carbonyl oxygen of these two residues. The side chain of Ser^{38} interacts with the N1 nitrogen of putrescine through the water molecule. Moreover, in PotF, the carboxyl oxygen atoms of Asp^{247} make both direct and water-mediated hydrogen bonds with the N1 atom of putrescine. Thus binding at the N1 site of putrescine in PotF is much stronger than that observed in PotD. This observation may explain the lower affinity of PotD than PotF for putrescine because the other interactions of the proteins with putrescine (i.e., the diaminobutane portion of spermidine) are very similar. In PotF, the position of the N1 atom of putrescine is strictly fixed, whereas in PotD, the N1 atom of spermidine is more flexible. To understand whether this difference may prevent binding of spermidine to PotF, we made a docking model of the spermidine molecule with a fixed position of its N1 amino group. There was no conformation that lacked steric hindrance with some amino acid residues in the PotF-binding sites. Thus we presume that the substrate selectivity of PotF is dominated by the unique hydrogen bond network with the N1 amino group, such that polyamines larger than putrescine can not fit into the PotF-binding cavity.

The crystal structures of PotD and PotF provide the first insight into the molecular mechanism by which proteins bind to and discriminate between different polyamines. The structural results, in combination with the mutational analyses, revealed that polyamine recognition could be achieved by the cooperation of multiple polar and


A
α5
α13
α4
C
βG
βF
βH
βE
α6
βI
α7
α12
α11
α2
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βD
βB
α10
α9
βA
βC
α8
α1
N
B
PotD
C-domain
Linker
N-domain
W229
E171
D168
Q327
W255
D257
Y85
S83
Y293
W34
Y37
T35
E36
N1
N2
N3
PotF
C-domain
Linker
N-domain
W244
E185
F276
D278
S87
S85
Y314
W37
Y40
S38
D39
S226
D247
N1
N2
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Fig. 3.

hydrophobic interactions. Experiments are now in progress to clarify the properties of the transmembrane proteins of polyamine-uptake systems.

The uptake of polyamines decreases following their accumulation in *E. coli*. One of the reasons for this is that spermidine inhibits the ATPase activity of PotA. Overexpression of PotD in *E. coli* also inhibited the uptake of spermidine and the synthesis of PotABCD messenger RNA (mRNA) *(32)*. A 50% inhibition of the transcription was observed with a molar ratio of approx 1:500 of template DNA/PotD in the presence of spermidine. PotD bound to regions –258 to –209 nucleotides upstream and +66 to +135 nucleotides downstream of the ATG initiation codon of the *potA* gene (Fig. 4). Binding of PotD to the downstream site was stimulated by spermidine. PotD exists as the PotD precursor in spheroplasts. Thus, the transcription of the *potABCD* operon is inhibited in vivo by the PotD precursor through its binding to two regions close to the transcriptional initiation site of the operon. To confirm that the PotD precursor functions as a regulator of the spermidine preferential uptake system operon, the number of PotD precursors in the spheroplast was estimated. Because the number of molecules of the major σ subunit of RNA polymerase (σ^{70}) is constant during the exponential phase of cell growth (about 700 molecules/cell) *(33)*, the number of PotD precursor was estimated by comparison with the number of RNA polymerase σ^{70} molecules. It was estimated to be 5000 to 25,000 PotD precursor molecules/cell in the spheroplast of *E. coli*-overexpressing PotD. These values can account for a 70–80% inhibition of spermidine uptake by excess PotD *(32)*. It appears that PotD, or more likely the PotD precursor, is a new type of transcriptional regulator.

PotE encoded by pPT71 and CadB belong to another class of polyamine transporter *(34–38)*. They consist of 12 transmembrane segments linked by hydrophilic segments of variable length with the NH_2- and the COOH-termini located in the cytoplasm. PotE and CadB can catalyze both the uptake at neutral pH and excretion at acidic pH of putrescine and cadaverine, respectively. Uptake of putrescine and cadaverine by PotE and CadB was dependent on the membrane potential, and the *Km* value for putrescine and cadaverine was 1.8 and 20.8 μ*M*. Excretion of putrescine and cadaverine was catalyzed by putrescine/ornithine and cadaverine/lysine antiporter activities of PotE and CadB. The *Km* value for putrescine and cadaverine for excretion was 73 and 303 μ*M*. The exchange ratio between putrescine (cadaverine) and ornithine (lysine) was 1:1. Excretion was increased by carbonyl cyanide *m*-chlorophenylhydrazone, which inhibits the membrane potential-dependent reuptake of putrescine and cadaverine. As for PotE protein, amino acid residues which are involved in both activities (Cys^{62}, Glu^{77}, Tyr^{92}, Trp^{201}, Glu^{207}, Cys^{210}, Cys^{285}, Cys^{286}, Trp^{292}, Tyr^{425}, and Glu^{433}) were located at the cytoplasmic surface

Fig. 3. Structure of PotD **(A)** and schematic drawing of PotD and PotF **(B)**. **(A)** The conserved sequence motif observed in maltodextrin-binding protein and PotD is shown in the circle, and the binding site for the spermidine molecule is marked by the side chains of the four acidic residues. **(B)** The polar and hydrophobic residues are drawn as ellipses and rectangles, respectively. The most crucial residues are shown in white ellipses and rectangles, and the other residues are shown is thick and thin shaded ellipses and rectangles. White circles represent water molecule. (Reproduced with permission from refs. *28* and *31*.)

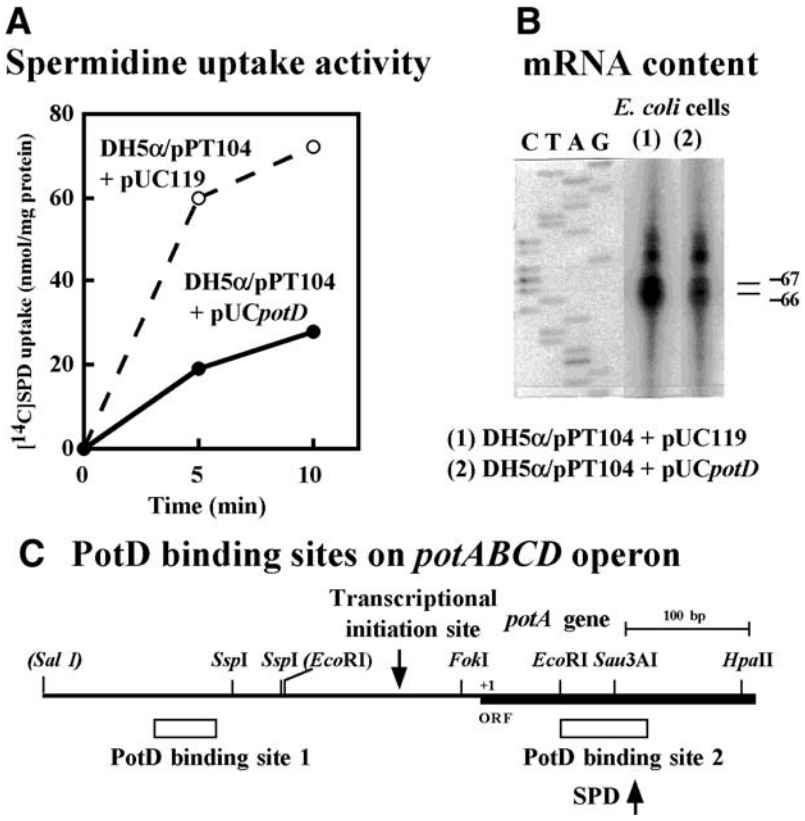

Fig. 4. **(A)** Inhibition of spermidine uptake by overexpression of PotD and PotD-binding sites on the *potABCD* operon. **(B)** The level of PotABCD mRNA was determined by primer extension. **(C)** PotD binding sites (–258 to –209 nucleotides upstream and 66 to 135 nucleotides downstream from the ATG initiation codon of the *potA* gene), which were determined by electrophoretic mobility shift assay, are shown as a box. (Reproduced with permission from ref. *32*.)

and the vestibule of the pore consisting of 12 transmembrane segments *(36,37)*. These results suggest that the active site of PotE is located at the cytoplasmic side.

The gene for *potE* (*cadB*) together with the *speF* (*cadA*) gene for inducible ornithine decarboxylase (ODC) (lysine decarboxylase) constitutes an operon. Cell growth of a polyamine-requiring mutant was stimulated slightly at neutral pH by the uptake activity and greatly at acidic pH by the antiporter activity. At acidic pH, these two operons were induced in the presence of ornithine and cadaverine, respectively. The induction of the operon caused neutralization of the extracellular medium and made possible the production of CO_2 and polyamines (Fig. 5). CO_2 produced by ornithine (lysine) decarboxylase contributes to the various metabolic pathways including nucleotide biosynthesis. Ornithine (lysine) decarboxylase also generates a pH gradient by consumption of a cytoplasmic proton. This process causes the increase in the level of ATP in cells.

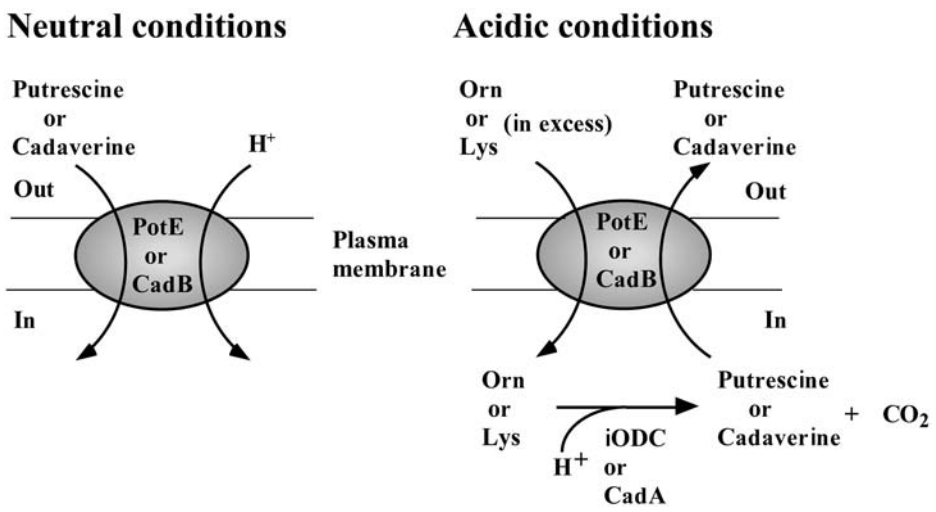

Fig. 5. Physiological functions of PotE and CadB in *Escherichia coli*. PotE, putrescine transporter; CadB, cadaverine transporter; iODC, inducible ornithine decarboxylase; CadA, lysine decarboxylase. iODC and CadA generate a pH gradient by consumption of a cytoplasmic proton in acidic conditions.

Because the *speF-potE* operon encoding inducible ODC and PotE is inducible at acidic pH, a protein involved in the enhancement of expression of the operon was sought. Using a fused gene containing the upstream sequence of the *speF-potE* operon and the open reading frame of β-galactosidase as a reporter gene, a clone that caused an increase of β-galactosidase activity at acidic pH was isolated. The clone was identified as a gene encoding RNase III *(39)*. Our results suggest that the initiation codon AUG and Shine-Dalgarno sequence are exposed on the surface of the mRNA by the RNase III processing of the 5′ untranslated region of mRNA. Although this is the first example of an RNase III-stimulated mRNA processing derived from *E. coli* gene, stimulation of the synthesis of λN protein and T7 0.3 gene protein by RNase III has been reported *(40,41)*. It is noteworthy that expression of the *speF-potE* operon is positively regulated at the posttranscriptional level.

3. Polyamine Transport in Yeast

Polyamine uptake in yeast is energy-dependent and the *Km* value for putrescine, spermidine, and spermine are 770, 8.3, and 18 μ*M*, respectively. A distinctive feature of the polyamine transport system in *Saccharomyces cerevisiae* is that it is strongly inhibited by Mg^{2+} *(42)*. Thus in a Mg^{2+}-limited medium, polyamines, especially spermine, overaccumulate in cells and become toxic for growth. We isolated a mutant (YTM22-8) whose growth was tolerant to spermine in a Mg^{2+}-limited medium. This mutant was defective in polyamine uptake and did not accumulate spermine. We obtained clones of two yeast genes that restored spermine sensitivity to YTM22-8. Those genes were *PTK1* and *PTK2*, which encode serine/threonine kinases *(43,44)*.

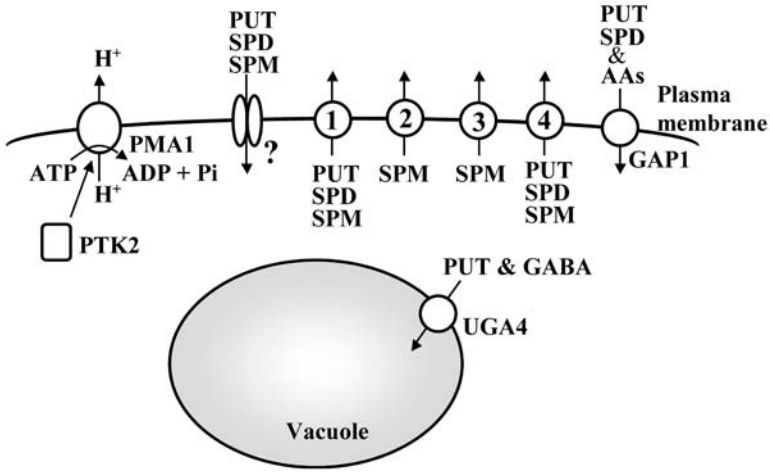

Fig. 6. Polyamine transporters in yeast. Polyamine-specific uptake proteins are not yet identified.

This suggests that spermine uptake in yeast is regulated by phosphorylation and dephosphorylation. Poulin and his coworkers also reported that another putative serine/threonine protein kinases, NPR1, essential for the reactivation of several nitrogen permeases, is involved in the activation of spermidine uptake in addition to PTK1 (= STK1) and PTK2 (= STK2) *(45,46)*. However, a gene for a polyamine-specific transporter on the plasma membrane of *Saccharomyces cerevisiae* has not yet been isolated. Recently, we found that UGA4, which is a transporter of 4-aminobutyric acid on the vacuolar membrane can take up putrescine *(47)*, and that GAP1, which is a general transporter of amino acids on the plasma membrane, can take up putrescine and spermidine *(48)*.

It has been reported that excretion of spermidine can be catalyzed by the *Bacillus subtilis* multidrug transporter Blt *(49)*. Thus we reasoned that the polyamine transporter in yeast may have some sequence similarity to Blt, and we searched for such amino acid sequence and proteins encoded by the yeast genome *(50)*. We could identify four genes that encode polyamine transport proteins TPO1 through TPO4 *(51,52)*. When expressed from chromosomes, these proteins were located on the plasma membrane *(53)*. When expressed from a multicopy vector, these proteins were mainly located on the plasma membrane, but with some localization on the vacuolar membrane *(51,52,54)*. Polyamine transport by TPO1 was dependent on pH. Uptake of polyamines occurred at alkaline pH (8.0), whereas excretion of polyamines occurred at acidic pH (5.0). Thus the function of TPO1 to 4 was similar to PotE and CadB. Because yeast cells usually grow at acidic pH, TPO1 to 4 function as excretion proteins for polyamines (Fig. 6). Among the four polyamine transporters, those encoded by *TPO2* and *TPO3* were specific for spermine, whereas those encoded by *TPO1* and *TPO4* recognized putrescine, spermidine, and spermine *(52)*. TPO1 consists of 586 amino acid residues *(50)* and has 12 putative transmembrane segments. Three glutamic acid residues (Glu^{207}, Glu^{324}, and Glu^{574}), which may

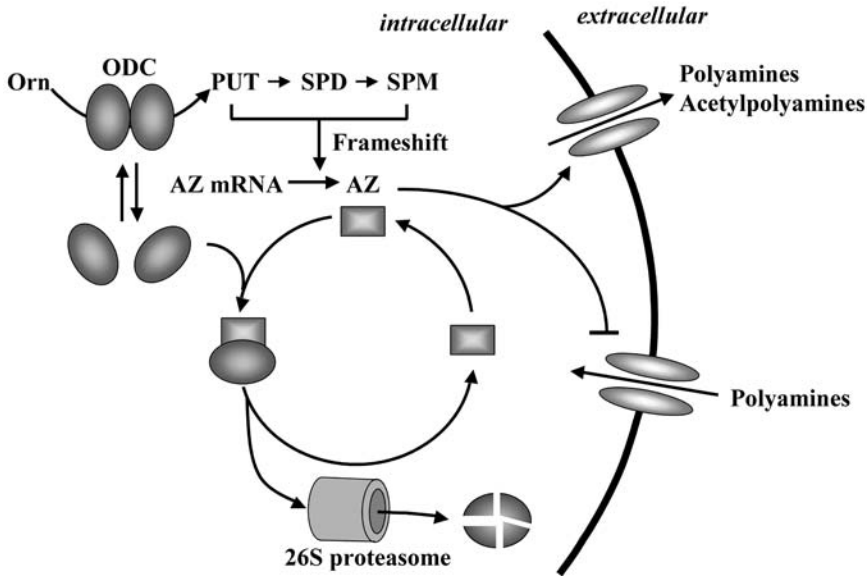

Fig. 7. Functions of antizyme. Polyamine transporters involved in uptake and excretion in mammalian cells are not yet identified.

interact with polyamines, are located in positions similar to those of the key residues in PotE (Glu^{77}, Glu^{207}, and Glu^{433}) *(52)*. When the amino acid sequence of TPO1 and PotE were compared, TPO1 possessed a longer hydrophilic NH_2-terminal region in which many serine and threonine residues are included, suggesting that polyamine transport is positively regulated by protein kinases. In support of this, phosphorylation of Ser^{19} by protein kinase C and that of Thr^{52} by casein kinase enhanced the transport activity of TPO1 *(54)*. Furthermore, the sorting of TPO1 protein to plasma membrane was enhanced by phosphorylation of Ser^{342} by cAMP-dependent protein kinases 1 and 2 *(54)*. Thus both uptake and excretion of polyamines are regulated by phosphorylation and dephosphorylation.

4. Polyamine Transport in Mammalian Cells

Polyamine uptake in mammalian cells is also energy dependent and the *Km* values for putrescine, spermidine, and spermine in bovine lymphocytes are 3.7, 0.38, and 0.23 μ*M*, respectively *(55)*. Polyamine uptake increases during cellular responses to proliferative stimuli, such as insulin or growth factors *(56,57)*, and its activity is also regulated by the intracellular levels of polyamines. Induction of polyamine transporter occurs when polyamine content becomes low *(55)*. However, a gene for polyamine-specific transporter has not yet been isolated.

Antizyme (AZ), which consists of 227 amino acid residues, is known to accelerate the degradation of ODC *(58,59)* and to inhibit the uptake of polyamines *(60,61)* (Fig. 7), shown by the following findings. Exposure of ODC-overproducing mouse

FM3A cells to macromolecular levels of spermidine or spermine caused the abnormal accumulation and toxicity of polyamines. When the cells were transfected with pMAMneoZ1-possessing AZ complementary DNA, a decrease in the amount of ODC, a decrease in the polyamine transport activity, and recovery of cell growth were observed *(61)*. The regulatory region of AZ necessary for the negative regulation of polyamine transport (peptides 119–144 and 211–216) overlapped with the binding site of ODC, but not with the region necessary for degradation of ODC (peptides 69–118) *(62)*. Furthermore, AZ enhanced the excretion of polyamines and acetylpolyamines *(63)*. Thus AZ plays important roles in the regulation of polyamine transport, as well as regulation of the level of ODC.

It has been reported that methylglyoxal *bis*(guanylhydrazone)-resistant Chinese hamster ovary cells show decreased polyamine transport activity *(64)*. Therefore, we looked for a gene to restore the polyamine transport activity. We found that TATA-binding associated factor 7 (TAF7) restored the transport activity of methylglyoxal *bis*(guanylhydrazone)-resistant Chinese hamster ovary cells, indicating a physiological function of TAF7 as a regulator for mammalian polyamine transport *(65)*. It has been reported recently that glypican-1 is a vehicle for polyamine uptake in mammalian cells *(10)*. Poulin and collaborators *(66)*, using a polyamine conjugated to a fluorescent dye, proposed a new idea that polyamines are imported first by a plasma membrane carrier and then sequestered into preexisting polyamine-sequestering vesicles or that they are directly captured by polyamine receptors undergoing endocytosis. Thus it is important to clarify whether a polyamine transporter(s) really exists for elucidation of polyamine transport in mammalian cells.

5. Concluding Remarks and Future Perspectives

In *E. coli*, some properties of the subunits of ABC transporters (substrate-binding protein, PotD and PotF, and ATPase, PotA) have been clarified. However, the properties of transmembrane proteins, such as PotB and PotC, still remain to be clarified. We hope that in the near future how the four subunits of the ABC transporter function together in polyamine transport will be clarified. Elucidation of the polyamine-binding site on PotD and PotF led to the identification of amino acid residues crucial for polyamine binding to the *N*-methyl-D-aspartate subtype of glutamate receptors *(66)*. As for the polyamine transport proteins consisting of 12 transmembrane segments linked by hydrophobic segments of variable length with the NH_2- and COOH-termini located in the cytoplasm (PotE and CadB), the physiological functions and amino acids residues involved in the activity have been elucidated. The tertiary structure of PotE and CadB will be clarified soon.

In eukaryotes, some properties of polyamine excretion proteins are clarified in yeast. All of those have 12 transmembrane segments. Thus the structure and functions of these proteins will be elucidated, referring to the information on PotE and CadB. However, the properties of polyamine uptake proteins are still unclear, although it is clear that polyamine uptake is negatively regulated by AZ. The demonstration of polyamine uptake through endocytosis mediated by glypican-1 is interesting. Nevertheless, it is still important to look for polyamine-specific transporters in eukaryotes.

Acknowledgments

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References

1. Cohen, S. S. (1998) *A Guide to the Polyamines.* Oxford University Press, Oxford, UK, pp. 1–543.
2. Igarashi, K. and Kashiwagi, K. (2000) Polyamines: Mysterious modulators of cellular functions. *Biochem. Biophys. Res. Commun.* **271,** 559–564.
3. Wallace, H. M., Fraser, A. V., and Hughes, A. (2003) A perspective of polyamine metabolism. *Biochem. J.* **376,** 1–14.
4. Seiler, N., Delcros, J. G., and Moulinoux, J. P. (1996) Polyamine transport in mammalian cells. An update. *Int. J. Biochem. Cell Biol.* **28,** 843–861.
5. Igarashi, K. and Kashiwagi, K. (1999) Polyamine transport in bacteria and yeast. *Biochem. J.* **344,** 633–642.
6. Igarashi, K., Ito, K., and Kashiwagi, K. (2001) Polyamine uptake systems in *Escherichia coli*. *Res. Microbiol.* **152,** 271–278.
7. Watanabe, S., Kusama-Eguchi, K., Kobayashi, H., and Igarashi, K. (1991) Estimation of polyamine binding to macromolecules and ATP in bovine lymphocytes and rat liver. *J. Biol. Chem.* **266,** 20,803–20,809.
8. Miyamoto, S., Kashiwagi, K., Ito, K., Watanabe, S., and Igarashi, K. (1993) Estimation of polyamine distribution and polyamine stimulation of protein synthesis in *Escherichia coli*. *Arch. Biochem. Biophys.* **300,** 63–68.
9. Williams, K. (1997) Interactions of polyamines with ion channels. *Biochem. J.* **325,** 289–297.
10. Belting, M., Mani, K., Jönsson, M., et al. (2003) Glypican-1 is a vehicle for polyamine uptake in mammalian cells. A pivotal role for nitrosothiol-derived nitric oxide. *J. Biol. Chem.* **278,** 47,181–47,189.
11. Igarashi, K., Kashiwagi, K., Hamasaki, H., et al. (1986) Formation of a compensatory polyamine by *Escherichia coli* polyamine-requiring mutants during growth in the absence of polyamines. *J. Bacteriol.* **166,** 128–134.
12. Kashiwagi, K., Hosokawa, N., Furuchi, T., et al. (1990) Isolation of polyamine transport-deficient mutants of *Escherichia coli* and cloning of the genes for polyamine transport proteins. *J. Biol. Chem.* **265,** 20,893–20,897.
13. Watson, N., Dunyak, D. S., Rosey, E. L., Slonczewski, J. L., and Olson, E. R. (1992) Identification of elements involved in transcriptional regulation of the *Escherichia coli cad* operon by external pH. *J. Bacteriol.* **174,** 530–540.
14. Meng, S. Y. and Bennett, G. N. (1992) Nucleotide sequence of the *Escherichia coli cad* operon: A system for neutralization of low extracellular pH. *J. Bacteriol.* **174,** 2659–2669.
15. Furuchi, T., Kashiwagi, K., Kobayashi, H., and Igarashi, K. (1991) Characteristics of the gene for a spermidine and putrescine transport system that maps at 15 min on the *Escherichia coli* chromosome. *J. Biol. Chem.* **266,** 20,928–20,933.
16. Pistocchi, R., Kashiwagi, K., Miyamoto, S., et al. (1993) Characteristics of the operon for a putrescine transport system that maps at 19 minutes on the *Escherichia coli* chromosome. *J. Biol. Chem.* **268,** 146–152.
17. Walker, J. E., Saraste, M., Runswick, M. J., and Gay, N. J. (1982) Distantly related sequences in the α- and β-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J.* **1,** 945–951.

18. Ames, G. F. (1986) Bacterial periplasmic transport systems: Structure, mechanism, and evolution. *Annu. Rev. Biochem.* **55,** 397–425.
19. Kashiwagi, K., Endo, H., Kobayashi, H., Takio, K., and Igarashi, K. (1995) Spermidine-preferential uptake system in *Escherichia coli*. ATP hydrolysis by PotA protein and its association with membranes. *J. Biol. Chem.* **270,** 25,377–25,382.
20. Kashiwagi, K., Miyamoto, S., Nukui, E., Kobayashi, H., and Igarashi, K. (1993) Functions of PotA and PotD proteins in spermidine-preferential uptake system in *Escherichia coli*. *J. Biol. Chem.* **268,** 19,358–19,363.
21. Kashiwagi, K., Innami, A., Zenda, R., Tomitori, H., and Igarashi, K. (2002) The ATPase activity and the functional domain of PotA, a component of the spermidine-preferential uptake system in *Escherichia coli*. *J. Biol. Chem.* **277,** 24,212–24,219.
22. Nikaido, K., Liu, P. Q., and Ames, G. F. (1997) Purification and characterization of HisP, the ATP-binding subunit of a traffic ATPase (ABC transporter), the histidine permease of *Salmonella typhimurium*. Solubility, dimerization, and ATPase activity. *J. Biol. Chem.* **272,** 27,745–27,752.
23. Nikaido, K. and Ames, G. F. (1999) One intact ATP-binding subunit is sufficient to support ATP hydrolysis and translocation in an ABC transporter, the histidine permease. *J. Biol. Chem.* **274,** 26,727–26,735.
24. Liu, C. E., Liu, P. Q., and Ames, G. F. (1997) Characterization of the adenosine triphosphatase activity of the periplasmic histidine permease, a traffic ATPase (ABC transporter). *J. Biol. Chem.* **272,** 21,883–21,891.
25. Hung, L. W., Wang, I. X., Nikaido, K., Liu, P. Q., Ames, G. F., and Kim, S. H. (1998) Crystal structure of the ATP-binding subunit of an ABC transporter. *Nature* **396,** 703–707.
26. Kühnau, S., Reyes, M., Sievertsen, A., Shuman, H. A., and Boos, W. (1991) The activity of the *Escherichia coli* MalK protein in maltose transport, regulation, and inducer exclusion can be separated by mutations. *J. Bacteriol.* **173,** 2180–2186.
27. Hunke, S., Landmesser, H., and Schneider, E. (2000) Novel missense mutations that affect the transport function of MalK, the ATP-binding-cassette subunit of the *Salmonella enterica serovar typhimurium* maltose transport system. *J. Bacteriol.* **182,** 1432–1436.
28. Sugiyama, S., Vassylyev, D. G., Matsushima, M., Kashiwagi, K., Igarashi, K., and Morikawa, K. (1996) Crystal structure of PotD, the primary receptor of the polyamine transport system in *Escherichia coli*. *J. Biol. Chem.* **271,** 9519–9525.
29. Sharff, A. J., Rodseth, L. E., Spurlino, J. C., and Quiocho, F. A. (1992) Crystallographic evidence of a large ligand-induced hinge-twist motion between the two domains of the maltodextrin binding protein involved in active transport and chemotaxis. *Biochemistry* **31,** 10,657–10,663.
30. Kashiwagi, K., Pistocchi, R., Shibuya, S., Sugiyama, S., Morikawa, K., and Igarashi, K. (1996) Spermidine-preferential uptake system in *Escherichia coli*. Identification of amino acids involved in polyamine binding in PotD protein. *J. Biol. Chem.* **271,** 12,205–12,208.
31. Vassylyev, D. G., Tomitori, H., Kashiwagi, K., Morikawa, K., and Igarashi, K. (1998) Crystal structure and mutational analysis of the *Escherichia coli* putrescine receptor. Structural basis for substrate specificity. *J. Biol. Chem.* **273,** 17,604–17,609.
32. Antognoni, F., Del Duca, S., Kuraishi, A., et al. (1999) Transcriptional inhibition of the operon for the spermidine uptake system by the substrate-binding protein PotD. *J. Biol. Chem.* **274,** 1942–1948.
33. Jishage, M. and Ishihama, A. (1995) Regulation of RNA polymerase sigma subunit synthesis in *Escherichia coli*: Intracellular levels of σ^{70} and σ^{38}. *J. Bacteriol.* **177,** 6832–6835.

34. Kashiwagi, K., Suzuki, T., Suzuki, F., Furuchi, T., Kobayashi, H., and Igarashi, K. (1991) Coexistence of the genes for putrescine transport protein and ornithine decarboxylase at 16 min on *Escherichia coli* chromosome. *J. Biol. Chem.* **266,** 20,922–20,927.
35. Kashiwagi, K., Miyamoto, S., Suzuki, F., Kobayashi, H., and Igarashi, K. (1992) Excretion of putrescine by the putrescine-ornithine antiporter encoded by the *PotE* gene of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **89,** 4529–4533.
36. Kashiwagi, K., Shibuya, S., Tomitori, H., Kuraishi, A., and Igarashi, K. (1997) Excretion and uptake of putrescine by the PotE protein in *Escherichia coli*. *J. Biol. Chem.* **272,** 6318–6323.
37. Kashiwagi, K., Kuraishi, A., Tomitori, H., et al. (2000) Identification of the putrescine recognition site on polyamine transport protein PotE. *J. Biol. Chem.* **275,** 36,007–36,012.
38. Soksawatmaekhin, W., Kuraishi, A., Sakata, K., Kashiwagi, K., and Igarashi, K. (2004) Excretion and uptake of cadaverine by CadB and its physiological functions in *Escherichia coli*. *Mol. Microbiol.* **51,** 1401–1412.
39. Kashiwagi, K., Watanabe, R., and Igarashi, K. (1994) Involvement of ribonuclease III in the enhancement of expression of the *speF-potE* operon encoding inducible ornithine decarboxylase and polyamine transport protein. *Biochem. Biophys. Res. Commun.* **200,** 591–597.
40. Kameyama, L., Fernandez, L., Court, D. L., and Guarneros, G. (1991) RNase III activation of bacteriophage lambda N synthesis. *Mol. Microbiol.* **5,** 2953–2963.
41. Dunn, J. J. and Studier, F. W. (1975) Effect of RNAase III, cleavage on translation of bacteriophage T7 messenger RNAs. *J. Mol. Biol.* **99,** 487–499.
42. Maruyama, T., Masuda, N., Kakinuma, Y., and Igarashi, K. (1994) Polyamine-sensitive magnesium transport in *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* **1194,** 289–295.
43. Kakinuma, Y., Maruyama, T., Nozaki, T., Wada, Y., Ohsumi, Y., and Igarashi, K. (1995) Cloning of the gene encoding a putative serine/threonine protein kinase which enhances spermine uptake in *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun.* **216,** 985–992.
44. Nozaki, T., Nishimura, K., Michael, A. J., Maruyama, T., Kakinuma, Y., and Igarashi, K. (1996) A second gene encoding a putative serine/threonine protein kinase which enhances spermine uptake in *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun.* **228,** 452–458.
45. Kaouass, M., Audette, M., Ramotar, D., et al. (1997) The STK2 gene, which encodes a putative Ser/Thr protein kinase, is required for high-affinity spermidine transport in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **17,** 2994–3004.
46. Kaouass, M., Gamache, I., Ramotar, D., Audette, M., and Poulin, R. (1998) The spermidine transport system is regulated by ligand inactivation, endocytosis, and by the Npr1p Ser/Thr protein kinase in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **273,** 2109–2117.
47. Uemura, T., Tomonari, Y., Kashiwagi, K., and Igarashi, K. (2004) Uptake of GABA and putrescine by UGA4 on the vacuolar membrane in *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun.* **315,** 1082–1087.
48. Uemura, T., Kashiwagi, K., and Igarashi, K. (2005) Uptake of putrescine and spermidine by Gap1p on the plasma membrane in *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun.* **328,** 1028–1033.
49. Woolridge, D. P., Vazquez-Laslop, N., Markham, P. N., Chevalier, M. S., Gerner, E. W., and Neyfakh, A. A. (1997) Efflux of the natural polyamine spermidine facilitated by the *Bacillus subtilis* multidrug transporter Blt. *J. Biol. Chem.* **272,** 8864–8866.
50. Goffeau, A., Barrell, B. G., Bussey, H., et al. (1996) Life with 6000 genes. *Science* **274,** 563–567.

51. Tomitori, H., Kashiwagi, K., Sakata, K., Kakinuma, Y., and Igarashi, K. (1999) Identification of a gene for a polyamine transport protein in yeast. *J. Biol. Chem.* **274,** 3265–3267.
52. Tomitori, H., Kashiwagi, K., Asakawa, T., Kakinuma, Y., Michael, A. J., and Igarashi, K. (2001) Multiple polyamine transport systems on the vacuolar membrane in yeast. *Biochem. J.* **353,** 681–688.
53. Albertsen, M., Bellahn, I., Krämer, R., and Waffenschmidt, S. (2003) Localization and function of the yeast multidrug transporter Tpolp. *J. Biol. Chem.* **278,** 12,820–12,825.
54. Uemura, T., Tachihara, K., Tomitori, H., Kashiwagi, K., and Igarashi, K. (2005) Characteristics of the polyamine transporter TPO1 and regulation of its activity and cellular localization by phosphorylation. *J. Biol. Chem.* **280,** 9646–9652.
55. Kakinuma, Y., Hoshino, K., and Igarashi, K. (1988) Characterization of the inducible polyamine transporter in bovine lymphocytes. *Eur. J. Biochem.* **176,** 409–414.
56. Pohjanpelto, P. (1976) Putrescine transport is greatly increased in human fibroblasts initiated to proliferate. *J. Cell. Biol.* **68,** 512–520.
57. Kano, K. and Oka, T. (1976) Polyamine transport and metabolism in mouse mammary gland. General properties and hormonal regulation. *J. Biol. Chem.* **251,** 2795–2800.
58. Murakami, Y., Tanaka, K., Matsufuji, S., Miyazaki, Y., and Hayashi, S. (1992) Antizyme, a protein induced by polyamines, accelerates the degradation of ornithine decarboxylase in Chinese-hamster ovary-cell extracts. *Biochem. J.* **283,** 661–664.
59. Murakami Y, Matsufuji S, Kameji T, et al. (1992) Ornithine decarboxylase is degraded by the 26S proteasome without ubiquitination. *Nature* **360,** 597–599.
60. Suzuki, T., He, Y., Kashiwagi, K., Murakami, Y., Hayashi, S., and Igarashi, K. (1994) Antizyme protects against abnormal accumulation and toxicity of polyamines in ornithine decarboxylase-overproducing cells. *Proc. Natl. Acad. Sci. USA* **91,** 8930–8934.
61. Mitchell, J. L., Judd, G. G., Bareyal-Leyser, A., and Ling, S. Y. (1994) Feedback repression of polyamine transport is mediated by antizyme in mammalian tissue-culture cells. *Biochem. J.* **299,** 19–22.
62. Sakata, K., Fukuchi-Shimogori, T., Kashiwagi, K., and Igarashi, K. (1997) Identification of regulatory region of antizyme necessary for the negative regulation of polyamine transport. *Biochem. Biophys. Res. Commun.* **238,** 415–419.
63. Sakata, K., Kashiwagi, K., and Igarashi, K. (2000) Properties of a polyamine transporter regulated by antizyme. *Biochem. J.* **347,** 297–303.
64. Mandel, J. L. and Flintoff, W. F. (1978) Isolation of mutant mammalian cells altered in polyamine transport. *J. Cell. Physiol.* **97,** 335–343.
65. Fukuchi, J., Hiipakka, R. A., Kokontis, J. M., Nishimura, K., Igarashi, K., and Liao, S. (2004) TATA-binding protein-associated factor 7 regulates polyamine transport activity and polyamine analog-induced apoptosis. *J. Biol. Chem.* **279,** 29,921–29,929.
66. Soulet, D., Gagnon, B., Rivest, S., Audette, M., and Poulin, R. (2004) A fluorescent probe of polyamine transport accumulates into intracellular acidic vesicles via a two-step mechanism. *J. Biol. Chem.* **279,** 49,355–49,366.
67. Masuko, T., Kashiwagi, K., Kuno, T., et al. (1999) A regulatory domain (R1-R2) in the amino terminus of the *N*-methyl-D-aspartate receptor: effects of spermine, protons, and ifenprodil, and structural similarity to bacterial leucine/isoleucine/valine binding protein. *Mol. Pharmacol.* **55,** 957–969.

26

Regulation of *S*-Adenosylmethionine Decarboxylase

Colin Hanfrey

1. Introduction

The biosyntheses of the essential polyamines spermidine and spermine are dependent on the activity of *S*-adenosylmethionine decarboxylase (AdoMetDC; EC 4.1.1.50). AdoMetDC catalyzes the conversion of *S*-adenosylmethionine (AdoMet) to *S*-adenosyl-5′-(3-methylthiopropylamine), otherwise known as decarboxylated AdoMet (dcAdoMet), which in turn donates its aminopropyl group to either putrescine for the synthesis of spermidine, or spermidine for the synthesis of spermine. The latter two reactions are catalyzed by the transferase enzymes, spermidine synthase and spermine synthase, respectively. In *Saccharomyces cerevisiae*, a null mutation in the *SPE2* gene, encoding AdoMetDC, conveys an absolute requirement for spermidine or spermine for growth *(1)*, and mouse blastocysts lacking a functional copy of the AdoMetDC gene die at the early stage of gastrulation unless supplied with spermidine *(2)*. As well as being the substrate for AdoMetDC, AdoMet is involved in many other essential biochemical processes in cells and is regarded as the major methyl donor in reactions catalyzed by methyltransferases. However, dcAdoMet is unable to fulfil these other functions, so AdoMetDC activity commits AdoMet to a role in polyamine biosynthesis.

AdoMetDC is synthesized as an inactive proenzyme (~38 kDa in humans) that undergoes an autocatalytic cleavage reaction to form the α and β subunits (~31 and ~7 kDa, respectively in humans) and the covalently bound pyruvoyl cofactor of the mature enzyme. In the decarboxylation reaction, the pyruvoyl group forms a Schiff base with AdoMet, providing an electron sink that facilitates removal of the α-carboxylate group. The negatively charged α-carbon of the substrate is then reprotonated, and Schiff base hydrolysis results in release of the product, dcAdoMet (Fig. 1). The most likely candidate for the role of proton donor in this reaction is the Cys-82 residue, because a C82A mutant protein has much reduced enzyme activity and exhibits a high incidence of incorrect protonation of the pyruvoyl group *(3)*.

Recent elucidations of the crystal structures of the human wild-type and mutated AdoMetDCs have provided profound insight into the evolution, activity, and regulation

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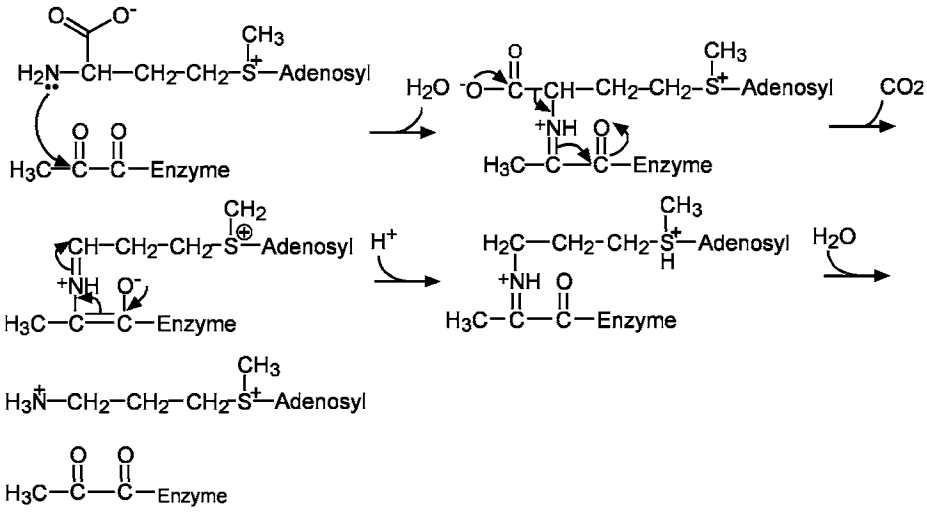

Fig. 1. Mechanism for AdoMetDC decarboxylation reaction. (Reprinted with permission from ref. *6*. © (2001) American Chemical Society.)

of the enzyme, and will undoubtedly lead to improved rational design of enzyme inhibitors for therapeutic intervention. The human AdoMetDC is an $(\alpha\beta)_2$ dimer, with each ($\alpha\beta$) monomer forming a novel four-layer $\alpha\beta\beta\alpha$ sandwich fold *(4)*. Two pieces of evidence suggest that the eukaryotic AdoMetDC probably evolved as a result of gene duplication. First, the human AdoMetDC exhibits an internal structural repeat, with the N- and C-terminal halves of the protein being related by an approximately twofold axis of symmetry *(4)*. Second, despite very little sequence homology, the prokaryotic *Thermotoga maritima* AdoMetDC has a structure very similar to that of the human AdoMetDC protomer and exhibits structural conservation of key active site residues previously identified in eukaryotic AdoMetDC *(5)*. The active site of the human AdoMetDC, located by the pyruvoyl residue, is within a cleft far from the dimer interface, and contains the catalytically important residues Glu-8, Glu-11, Ser-69, Ser-229, His-243, and the aforementioned Cys-82 *(4)*. Crystal structures of the human AdoMetDC complexed with substrate analogs indicate that Cys-82, Ser-229, and His-243 are positioned close to the methionyl group of the substrate, and the side chains of Phe-7, Phe-223, and Glu-247 serve to bind and position the substrate within the active site *(6)*. Interestingly, the AdoMet substrate is bound in the *syn* orientation, despite the fact that the *anti* conformation probably predominates in solution *(7)*.

Reflecting its key role in both polyamine biosynthesis and AdoMet metabolism, AdoMetDC is a highly regulated enzyme, with changes in enzyme activity being achieved via control at the levels of transcription, messenger RNA (mRNA) stability, translation, proenzyme processing, enzyme activation, enzyme inactivation, and enzyme degradation. Furthermore, the mode and degree of regulation of AdoMetDC activity is dependent on cell type and growth rate, as well as cellular polyamine levels.

This chapter will focus on the regulation of the mammalian AdoMetDC; although where findings in other species are of interest or relevance, they will be discussed. The reader is also referred to previously published articles that provide comprehensive reviews of AdoMetDC regulation *(8–10)*.

2. Regulation by Transcription and Posttranscriptional mRNA Stabilization

Most treatments that result in an increase in AdoMetDC activity appear to achieve this, at least partially, by raising AdoMetDC mRNA levels, although changes in mRNA are seldom sufficient to account for the total observed changes in activity *(8)*. An increase in steady-state mRNA level can reflect an upregulation of transcriptional activity or increased stabilization of the mRNA or a combination of both. However, distinctions between these two processes have been made in only a few experimental studies on AdoMetDC mRNA accumulation. The human AdoMetDC gene consists of nine exons and eight introns, covering approx 22 kb of chromosome 6. The functionally defined promoter region (–3158 to +1) contains DNA recognition motifs for transcription factors AP-1, AP-2, SP-1, and CREB, and a number of half sites for interaction with glucocorticoid, androgen, and estrogen receptors *(11)*.

Of the polyamines, spermidine appears to have the greatest influence on AdoMetDC mRNA accumulation. For example, in rat prostate and in cultured mouse cells, depletion of polyamines by treatment with the ornithine decarboxylase (ODC) inhibitor, α-difluoromethylornithine, led to increased AdoMetDC activity that was accompanied by an increase in AdoMetDC mRNA; these changes were reversed by administration of spermidine. However, the specific spermine synthase inhibitor, $AdoS^{+}(CH_3)_{2,}$ produced a larger increase in activity but a much smaller change in mRNA *(12)*. Similarly, in HT29, CHO, and COS-7 cell lines, AdoMetDC activity was inversely related to polyamine content, but only spermidine affected AdoMetDC mRNA levels *(13)*.

Studies with oncogene-transformed cells revealed that AdoMetDC mRNA expression is regulated by different means according to the nature of the oncogene expressed and the malignant potential of the cells. In H-ras-transformed mouse cells, the AdoMetDC transcription rate was increased, but there was no change in mRNA stability, whereas in raf-transformed cells, the opposite was true *(14)*. H-ras-transformed cells exposed to epidermal growth factor, basic fibroblast growth factor, transforming growth factor β(1), or platelet derived growth factor exhibited an increase in AdoMetDC activity, but only epidermal growth factor and basic fibroblast growth factor caused increased AdoMetDC mRNA, apparently by enhanced mRNA stabilization *(15)*.

Insulin-mediated increases in AdoMetDC mRNA in H-ras-transformed mouse cells were brought about exclusively by increased transcription in a cell line capable of forming benign tumors, but by a combination of increased transcription and stabilization of the mRNA in a cell line that exhibited malignant characteristics *(16)*. In rat hepatoma cells, treatment with insulin led to increased AdoMetDC activity caused by an increase in transcription rate, and reporter gene assays indicated that the 377-bp promoter of the rat AdoMetDC contains insulin-responsive elements *(17)*.

During the cell cycle, both AdoMetDC activity and mRNA content double during the G_1/S transition, indicating transcriptional control or mRNA stabilization during G_1. However, regulation of AdoMetDC activity during S and G_2 appear to be achieved at the translational or posttranslational level *(18)*.

3. Translational Regulation

The translational efficiency of AdoMetDC mRNA is regulated by growth stimulation, changes in intracellular levels of spermidine and spermine, and according to cell type. In vitro experiments with a reticulocyte lysate system demonstrated that translation of AdoMetDC mRNA was maximal when polyamines had been removed from the lysate by gel filtration, and that adding back 80 μ*M* spermine or 0.8 m*M* spermidine substantially reduced AdoMetDC synthesis, while having no effect on total protein synthesis *(19)*. Treatment of Swiss 3T3 fibroblasts with α-difluoromethylornithine caused AdoMetDC mRNA to become associated exclusively with large polysomes, indicating that polyamines mediate their effect via a mechanism involving translation initiation *(20)*.

The scanning model of eukaryotic translation initiation predicts that the 43S preinitiation complex assembles at the 5′ cap of the mRNA, and then scans the 5′ leader linearly until it encounters an AUG codon in a sequence context that is favorable for initiation, whereupon other factors, including the 60S ribosomal subunit, are recruited, and the first peptide bond is formed *(21)*. The long (~330 nucleotides) 5′ leader sequences of mammalian AdoMetDC mRNAs contain potential obstacles to this scanning process *(11,12)*. First, the predicted secondary structure of the human AdoMetDC mRNA 5′ leader is relatively stable, with a Δ*G* value of –69 kcal/mol *(22)*, which could inhibit downstream initiation *(23)*. However, experimental evidence suggests that this secondary structure plays no part in the translational regulation of AdoMetDC. In fact, in COS-7 cells transfected with the AdoMetDC cDNA, removal of only the 27 nucleotides nearest the 5′ end of the leader was sufficient to alleviate the repressive effect on translation of the AdoMetDC open reading frame *(22)*. Also, initiation factor eIF-4E is known to be a limiting factor in the translation of mRNAs that have extensive secondary structure within their 5′ leaders, but overexpression of eIF-4E in 3T3 cells had no effect on AdoMetDC expression *(24)*.

The second potential obstacle to ribosome scanning in the AdoMetDC mRNA is a short (21 nucleotides) upstream open reading frame (uORF), located approx 14 nucleotides downstream from the 5′ cap site *(25)*. There are several examples in which the presence of a uORF has been shown to regulate translation of the downstream cistron (reviewed in *(26–28)*). For the uORF to exert a regulatory role, its AUG codon must be recognized by the scanning preinitiation complex, and this depends on the sequence context of the AUG, its proximity to the 5′ cap, and the degree of secondary structure preceding it *(29)*. Constructs in which the AdoMetDC uORF had been eliminated by altering the AUG to CGA led to an increase in AdoMetDC expression comparable to that observed when virtually the entire 5′ leader was removed *(22)*. Furthermore, depletion of spermine led to a 2.5-fold increase in expression when the uORF had been removed, compared with a fivefold increase observed with the wild-type 5′ leader,

indicating that the uORF plays a role in the translational response to spermine. Similar experiments were performed with AdoMetDC 5′ leader–luciferase constructs, and, in this case, removal of the uORF did not result in an increase in luciferase expression when spermine was depleted, suggesting that the 2.5-fold observed increase in AdoMetDC expression in the absence of the uORF resulted from stabilization of the enzyme rather than any effect on translation *(22)*. Introduction of the uORF sequence into the 5′ leader of the human growth hormone mRNA was sufficient to impart polyamine-regulated translation on this mRNA *(30)*.

The AdoMetDC uORF encodes a predicted hexapeptide with the sequence Met-Ala-Gly-Asp-Ile-Ser (commonly referred to as MAGDIS). Altering the encoded amino acid at position four, five, or six abolished the negative regulatory effect and the polyamine responsiveness of the uORF, whereas synonymous changes to the nucleic acid sequence did not result in a loss of regulation *(30,31)*. Hill and Morris *(31)* also established that the uORF only functions in *cis*. More detailed analysis of the AdoMetDC uORF sequence specificity revealed that the requirements at positions four and five are stringent, with only Asp being fully functional at position four, and only Val being an adequate substitute for Ile at position five. Several amino acid substitutions at position six were possible without affecting uORF function, but placing a termination codon at position six, or extending the peptide by a single Ala residue resulted in a loss of function *(32)*. Although the *Saccharomyces cerevisiae* AdoMetDC mRNA does not possess a uORF and is not translationally regulated in response to polyamines, the mammalian uORF conferred sequence-dependent, polyamine-regulated translation when expressed in *S. cerevisiae (32,33)*. Therefore, it is likely that regulation is achieved via an interaction between the MAGDIS peptide (specifically the fourth and fifth amino acids) and a component of the translation machinery that is conserved between species. These observations place the AdoMetDC uORF within a relatively exclusive group of so called sequence-dependent uORFs, in which the uORF-encoded peptide is responsible for the observed regulation, presumably via an interaction with the translation machinery that leads to "ribosome stalling" on the mRNA *(26–28)*.

A key feature of the "ribosome stalling" model of polyamine-regulated AdoMetDC translation is the prediction that the uORF is translated, but for such a small peptide product, this is technically difficult to demonstrate. Raney et al. *(34)* overcame this problem by devising a new analytical procedure. They added an unlabeled synthetic peptide to completed in vitro translation reactions to act as a carrier for the small ^{35}S-labeled products. Peptides were purified by sequential high-performance liquid chromatography and thin-layer chromatography (TLC), and the ^{35}S-labeled hexapeptide was quantified by phosphorimaging of the TLC plates. Using this approach, Raney et al. *(34)* were able to show that translation of repressive uORF peptides (i.e., MAGDIS and MAGDVS) was more sensitive to increases in spermidine and spermine than was the synthesis of nonrepressive peptides (MAGEIS, MAGDLS, and MAGDI).

A second prediction of the "ribosome stalling" model of AdoMetDC regulation is that an interaction between the nascent MAGDIS peptide and a component of the translation machinery leads to a ribosome being halted on the mRNA, forming a physical block to scanning ribosomes and thus preventing access to the downstream

AdoMetDC-encoding cistron *(32)*. A technique known as "toeprinting" enables mapping of the 3′ boundaries of stalled ribosomes on mRNAs *(35)*. Briefly, a primer extension assay is performed, but with an in vitro translation reaction rather than purified RNA as template. The presence of a bound ribosome (or other factor) on an mRNA will inhibit extension by the reverse transcriptase enzyme, resulting in a truncated product, or "toeprint." These products are resolved on a denaturing gel alongside a sequence ladder for the mRNA of interest, enabling the sizes and positions of the toeprints to be determined. Law et al. *(36)* used toeprinting to detect ribosomes paused in the vicinity of the termination codon of the MAGDIS uORF and to show that these interactions were stabilized specifically by polyamines. Stalled ribosomes were also detected with altered nonrepressive uORFs, but the toeprints were significantly lower in intensity than those with MAGDIS. The authors proposed that these observations explain the sensitivity of MAGDIS translation to polyamines *(34)*, because the small size of the MAGDIS uORF means that a ribosome paused at its termination codon would also occlude the initiation site. In addition, Law et al. *(36)* were able to demonstrate that, under conditions in which the ribosome pause was stable (i.e., high spermidine levels), there was a reduction in the number of ribosomes reaching the AdoMetDC initiation codon, thereby providing a link between polyamine-regulated ribosome stalling and AdoMetDC translation.

Eukaryotic translational termination is triggered by the presence of one of the three termination codons (UAA, UAG, or UGA) in the A site of the ribosome. Eukaryotic release factor 1 then binds the termination signal, the ester bond between the transfer RNA (tRNA) located in the P site and the nascent polypeptide chain is hydrolyzed, and the ribosome, tRNA, and protein dissociate from the mRNA *(37)*. In in vitro translation reactions programmed with the AdoMetDC mRNA, a ribosome-associated, ^{35}S-labeled complex that was sensitive to RNase A and proteinase K was detectable *(38)*. The ^{35}S-labeled peptide component of this molecule comigrated with the MAGDIS peptide on a TLC plate, and synonymous mutations in the final serine codon of the uORF altered the electrophoretic mobility of the complex, suggesting that the RNA component was a $tRNA^{SER}$ molecule. Based on this evidence, the authors concluded that this complex was the nascent MAGDIS peptide linked to the tRNA that decodes the final serine codon (MAGDIS–$tRNA^{SER}$). The MAGDIS–$tRNA^{SER}$ complex was stabilized by elevated polyamines in the in vitro translation mixture, and mutations in the critical fourth and fifth amino acid positions of the uORF resulted in a less abundant complex of reduced stability. Therefore, the regulated step in AdoMetDC uORF translation termination occurs after formation of MAGDIS–$tRNA^{SER}$, but before hydrolysis of the peptidyl-tRNA bond. Exactly how polyamines modulate this regulation remains to be elucidated.

The AdoMetDC mRNAs of plant species have a highly conserved arrangement of uORFs in their 5′ leaders. A tiny uORF, consisting of three to four codons, overlaps by one nucleotide with a downstream small uORF of between 50 and 54 codons, the predicted amino acid sequence of which is very highly conserved among plant species *(39)*. The small uORF is a sequence-dependent repressor of downstream AdoMetDC translation, and the tiny uORF is required to impart polyamine sensitivity on this

regulation (*40*; Hanfrey, C., Elliott, K. A., Franceschetti, M., Mayer, M. J., Illingworth, C., and Michael, A. J., unpublished data). The plant tiny uORF/small uORF arrangement and the mammalian MAGDIS uORF are so different in sequence, size, and position on their respective mRNA 5′ leaders, that they may represent an intriguing case of convergent evolution.

4. Proenzyme Processing

AdoMetDC belongs to a class of pyruvoyl-dependent enzymes that includes aspartate decarboxylase, phosphatidylserine decarboxylase, and histidine decarboxylase *(41)*. The catalytic activities of these enzymes rely on a covalently bound pyruvoyl group rather than the pyridoxal-5′-phosphate cofactor more commonly employed by amino acid decarboxylases. Pyruvoyl enzymes are synthesized as an inactive proenzyme that undergoes an apparently autocatalytic internal cleavage reaction to yield two subunits (in the case of AdoMetDC, a larger C-terminal-derived α subunit and a smaller N-terminal-derived β subunit). In mammalian AdoMetDC, the cleavage site is located between Glu-67 and Ser-68 within the conserved motif YVLSESS, and the pyruvoyl group is formed by conversion of the resulting serine residue at the amino terminus of the α subunit *(42)*.

The chemical mechanism for formation of the AdoMetDC pyruvoyl involves nucleophilic attack by the hydroxyl group of Ser-68 at the carbonyl carbon atom of Glu-67, resulting in the formation of an oxyoxazolidine intermediate. This intermediate undergoes an N→O acyl rearrangement to form an ester intermediate, which in turn undergoes a β elimination reaction to form the α subunit with a dehydroalanine residue at its N-terminus, and the β subunit. The dehydroalanine is then converted to pyruvoyl with the loss of ammonia via the formation of imine and carbinoalamine intermediates *(43)* (Fig. 2).

The crystal structure of wild-type AdoMetDC in its processed form revealed that residue Glu-67 remains close to the pyruvate residue, indicating that the protein does not undergo a major conformational change after cleavage, and hence conserved residues within the active site pocket are also candidates for a role in processing *(4)*. Three such residues are Cys-82, Ser-229, and His-243, and mutation of these amino acids resulted in impaired proenzyme processing. Mutant C82A processed approximately 10 times more slowly than the wild-type enzyme *(44)*. Mutant S229A did not process, S229C processed at a much reduced rate, and S229T processed normally, suggesting that the hydroxyl group of Ser-229 is critical to the reaction *(45)*. Mutant H243A cleaved very slowly, with the majority of the resulting α subunit having an N-terminal serine rather than a pyruvoyl, but cleavage was accelerated by addition of hydroxylamine *(45)*. This latter result is indicative of cleavage occurring via hydrolysis of the ester intermediate rather than β elimination, and solving of the crystal structure of the H243A mutant revealed that the protein is indeed trapped in the ester form, confirming that it is the β elimination step that is prevented in this mutant *(46)*. Based on these observations and modeling of the oxyoxazolidine intermediate from the crystal structure of the nonprocessing S68A mutant, Tolbert et al. *(43)* proposed a processing mechanism in which the formation of the oxyoxazolidine intermediate is promoted by

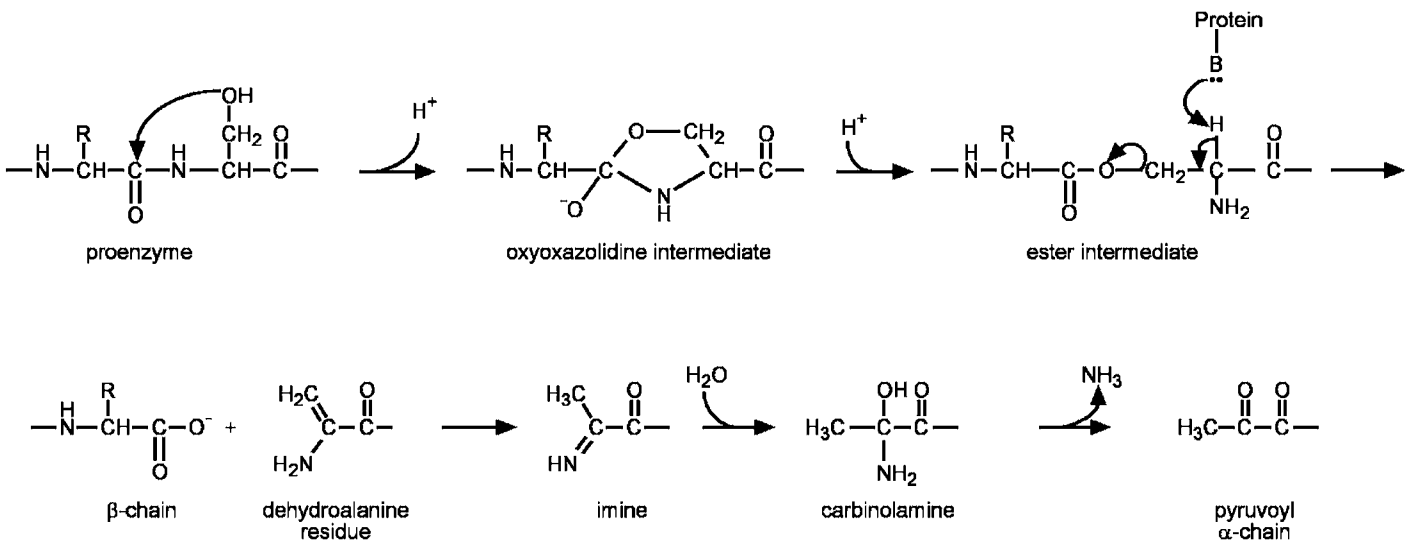

Fig. 2. Mechanism for AdoMetDC pyruvoyl group formation. (Reprinted with permission from ref. *43*. © (2003) American Chemical Society.)

a hydrogen bond from Cys-82 and then stabilized by a hydrogen bond from Ser-229. Donation of a proton by His-243 to the nitrogen atom of the oxyoxazolidine intermediate then leads to formation of the ester intermediate. Because the H243A mutant enzyme is able to form the ester intermediate *(46)*, Tolbert et al. *(43)* postulated that a water molecule may act as a substitute proton donor in this mutant. The suggested mechanism for the next step in the processing reaction is abstraction of the H^{α} proton of Ser-68 by the His-243 side chain, resulting in cleavage of the peptide chain and formation of the dehydroalanine residue *(46)*.

The rate of the mammalian AdoMetDC processing reaction is accelerated by the addition of putrescine *(47)*, and the four acidic residues Glu-11, Asp-174, Glu-178, and Glu-256 are necessary for this stimulation *(48)*. A single putrescine molecule was bound to each monomer of the H243A mutant, within a cluster of buried negatively charged residues between the two β sheets, about 15–20 Å from the active site *(46)*. The presence of a single bound putrescine molecule was unexpected for two reasons. First, putrescine had not been added during the crystallization process, leaving the *Escherichia coli* protein expression system as the most likely source. Second, kinetics data from experiments with putrescine and two substituted analogs had strongly suggested two or more binding sites for putrescine *(8)*. The single putrescine molecule observed in H243A forms direct interactions with the side chains of Glu15, Asp174, and Thr176, and indirect water-mediated interactions with side chains of Glu15, Ser113, Glu178, and Glu256. Furthermore, this putrescine-binding site is linked to the active site by a network of hydrogen bonds, involving residues Glu-11, Lys-80, and His-243 *(46)*. These observations led Ekstrom et al. *(46)* to postulate that putrescine binding serves to balance the negative charges of the binding site, thereby repositioning critical active site residues, either by causing a shift in the relative orientations of the β sheets, or by charge transmission via the hydrogen bond network, or both. However, the structure of the proenzyme (i.e., the S68A nonprocessing mutant) and the modeled structure of the oxyoxazolidine intermediate demonstrate that neither Glu-11 nor Lys-80 interact with Cys-82 or Ser-229, leading to the conclusion that putrescine influences processing mainly via an effect on His-243 *(43)*.

Unlike the mammalian enzyme, plant AdoMetDCs undergo very rapid autoprocessing and do not require putrescine for an optimal processing rate *(48)*. The crystal structure of the potato AdoMetDC provides an explanation for this difference *(49)*. Two amino acid changes in the buried putrescine-binding site, Leu-13/Arg-18 and Phe-111/Arg114, indicate that two arginine residues could substitute for putrescine in balancing the negative charge of the site. Unlike putrescine, which binds reversibly to the mammalian enzyme, these arginine residues are always present, so the enzyme will always be in the activated state, thus explaining the apparent rapid autoprocessing of plant AdoMetDCs. The potato AdoMetDC structure also revealed that there is a very similar network of hydrogen bonds between the charged site and the active site as seen in the human AdoMetDC, supporting the proposed role of this network in transmission of the putrescine/arginine effect. Interestingly, the potato AdoMetDC does not form a dimer, but rather a monomer (αβ). This is probably owing to substitutions of several residues that, in the human AdoMetDC, form interactions across the dimer interface;

but it may also have significance in regulation of the respective enzymes because disruption of the human dimer interface would probably make the putrescine-binding site more exposed *(49)*.

5. Enzyme Activation

As well as stimulating the rate of proenzyme processing, putrescine stimulates catalytic activity of the processed enzyme by reducing the K_m for AdoMet *(8)*. Hence supply of putrescine is linked to the production of dcAdoMet, the other precursor for spermidine production. Site-directed mutagenesis experiments demonstrated that residues Glu-178 and Glu-256 are critical for putrescine stimulation of catalytic activity and for proenzyme processing of mammalian AdoMetDC, but because the E11Q mutant was completely inactive, it was not possible to assess the importance of Glu-11 *(50)*. The binding of putrescine to AdoMetDC and the residues involved are discussed in the preceding section. It has been suggested that the observed effect of putrescine on enzyme activity may be achieved by the same hydrogen-bonding effects or structural shifts as those postulated to be involved in processing *(46)*. However, it should be noted that results of experiments with various site-directed mutants led Stanley *(8)* to conclude that putrescine stimulation of processing is achieved by forcing a particular proenzyme conformation, for which the negatively charged residues of the binding site are required, but putrescine stimulation of catalytic activity occurs by a permissive effect, whereby putrescine interaction with the same negatively charged residues allows a conformational change that lowers the K_m for the substrate. Furthermore, although 4-aminobutanol stimulated proenzyme processing, it had no effect on enzyme activity *(46)*. To summarize, it appears that discrepancies still exist between gathered biochemical data and putrescine-binding characteristics observed at the structural level, and that further studies in both aspects will be required to elucidate the precise mechanisms involved in putrescine-stimulated processing and catalytic activity.

In common with plants, the causal agent of Chagas disease, *Trypanosoma cruzi*, has an AdoMetDC that is fully processed in the absence of putrescine, and has a Leu-13/Arg-13 substitution when compared with the human enzyme. However, the *T. cruzi* enzyme does bind putrescine and, unlike plant AdoMetDCs, its catalytic activity is stimulated by putrescine. Site-directed mutagenesis studies revealed that Asp-174 (numbered according to the human AdoMetDC sequence) is the only common residue that is involved in putrescine binding in both the human and the *T. cruzi* AdoMetDCs, suggesting that the binding sites in the respective enzymes at most only partially overlap *(51)*. Plant AdoMetDCs have a Met, Val, or Ile substitution at the 174-equivalent position.

The AdoMetDC of the malarial parasite, *Plasmodium falciparum*, is unusual in that it forms part of a bifunctional polypeptide together with ODC. The N-terminal AdoMetDC domain is linked to the C-terminal ODC domain by a hinge region *(52)*. Consequently, both enzymes are transcribed and translated together. However, the AdoMetDC domain is still processed by cleavage at the conserved LSESS site, and the active ODC/AdoMetDC enzyme forms a heterotetramic complex *(52,53)*. The *P. falciparum* AdoMetDC lacks the Asp-174 residue, and is not stimulated by putrescine,

but an advantage of the bifunctional enzyme is that putrescine production and AdoMet decarboxylation are linked *(53)*.

The catalytic activity of the AdoMetDC of the free-living nematode, *Caenorhabditis elegans* is stimulated by putrescine, but unusually, it is also activated by spermidine. The biological significance of this apparent feedback stimulation is unknown *(54)*.

6. Enzyme Inactivation and Degradation

AdoMetDC has a relatively short half-life (1–2 h), which varies with cell type, and is inversely related to cellular levels of spermidine and spermine *(9)*. Many short-lived proteins in eukaryotic cells are degraded by the ubiquitin/proteasome pathway *(55,56)*. The adenosine triphosphate (ATP)-dependent ubiquitination process results in joining of a ubiquitin polypeptide to the protein substrate via an isopeptide bond between a lysine side chain of the substrate and the C terminus of ubiquitin. This modification targets the protein for degradation by the 26S proteasome, which uses energy from ATP hydrolysis to unfold and then hydrolyze the substrate protein, whereas the ubiquitin is recycled. In COS-7 cells, AdoMetDC antigen has a half-life of 1.9 h, but ATP depletion or treatment with specific 26S proteasome inhibitors (MG-132, lactacystin, or clastolactacystin β-lactone) completely stabilized the enzyme *(57)*. ODC, which catalyzes the other key step in polyamine biosynthesis, was the first reported example of a protein that undergoes degradation by the 26S proteasome without ubiquitination. Instead, ODC is targeted for degradation by binding of the inhibitory antizyme (AZ) protein, the synthesis of which is induced by polyamines *(58)*. Cellular levels of ODC and AdoMetDC tend to follow similar patterns *(10)*. However, AdoMetDC did not coprecipitate with AZ antibodies, and AZ did not coprecipitate with AdoMetDC antibodies, suggesting that AdoMetDC does not share the same route to degradation as ODC *(57)*. In fact, when the 26S proteasome was inhibited there was an accumulation of polyubiquitin-tagged AdoMetDC, implying that AdoMetDC degradation is ubiquitin dependent *(57)*.

During the cell cycle, the increase in AdoMetDC activity observed during G_1/S transition was accompanied by an increase in half-life of the enzyme activity *(59)*, as well as an increase in AdoMetDC mRNA *(18)*. However, during S phase, when AdoMetDC activity is increased, activity half-life remained constant *(59)*.

Pyruvoyl enzymes can be irreversibly inactivated by a process known as substrate-mediated transamination *(41)*. In transamination of AdoMetDC, incorrect protonation of the pyruvate group instead of the α-carbon of AdoMet leads to release of the product in an aldehyde form, and conversion of the pyruvate to an alanine *(3)*. A substantial portion of isolated AdoMetDC from *E. coli*, *Salmonella typhimurium*, and *Saccharomyces cerevisiae* was found to have an alanine instead of a pyruvoyl group *(60)*. It has been suggested that transaminated (inactive) AdoMetDC may be a target for rapid degradation *(9)*; an idea supported by the report that the AdoMetDC inhibitor AbeAdo (5′-{[(*Z*)-4-amino-2-butenyl]methylamino}-5′-deoxyadenosine), which stimulates transamination *(61)*, reduced AdoMetDC half-life from 2.7 to 1.5 h in COS-7 cells *(57)*. Furthermore, depletion of substrate AdoMet, which is predicted to reduce the incidence

of transamination, led to an increase in AdoMetDC half-life *(57)*. If, as suggested, enzyme inactivation by transamination is the cause of the observed rapid turnover of AdoMetDC *(9)*, then inactivated enzyme would be predicted to be degraded more rapidly than active enzyme. As previously stated, inhibition of the 26S proteasome in COS-7 cells led a complete stabilization of the AdoMetDC antigen. However, AdoMetDC activity declined at a rate similar to that in untreated control cells *(57)*. If active and inactive enzyme were degraded at the same rate, then the loss of activity would be greater in control cells because the degradation of antigen (and hence active enzyme) would be occurring in these cells. Therefore, it seems likely that transaminated AdoMetDC is preferentially degraded. Recently, the shortest half-life for a eukaryotic AdoMetDC was recorded for the protozoan *Crithidia fasciculata* (estimated at 3 min). However, transamination of this enzyme appears to be a relatively rare event, and the mechanism responsible for this rapid turnover is unclear *(62)*.

Nitric oxide, an important second messenger molecule in a range of physiological processes, inactivates AdoMetDC by *S*-nitrosylation of the active site residue Cys-82. Combined with its inactivation of ODC by a similar mechanism, this observation may partly explain the antiproliferative effects of nitric oxide *(63)*.

7. Concluding Remarks

AdoMetDC catalyzes a key step in polyamine biosynthesis, and its activity competes with many other essential biochemical processes for the cellular pool of AdoMet. It is therefore not surprising that AdoMetDC is a very highly regulated enzyme, with its synthesis and activity being controlled at multiple steps, and in response to cell growth rate and to cellular levels of precursors (putrescine and AdoMet) and downstream products (spermidine and spermine). AdoMetDC mRNA accumulation is regulated according to spermidine concentration, as well as certain growth factors and hormones; mRNA translation is controlled via a complex interaction between polyamines, an uORF-encoded small peptide, and a conserved element of the translation machinery; putrescine stimulates proenzyme processing and activates the mature enzyme; and levels of AdoMet and spermidine and spermine influence enzyme stability. Thus AdoMetDC makes a challenging but useful model for studying regulation of gene expression. AdoMetDC's key role in polyamine biosynthesis has led to it becoming a target for chemotherapeutic intervention in treating cancers and parasite infections *(64,65)*. However, many of the AdoMetDC inhibitors have limited efficacy in vivo and produce side effects that make them unsuitable for use. Findings of studies such as those described in this chapter are providing an ever clearer picture of AdoMetDC structure, function, and regulation, which will certainly aid the design of more specific and more active inhibitors.

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References

1. Balasundaram, D., Tabor, C. W., and Tabor, H. (1991) Spermidine or spermine is essential for the aerobic growth of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **88,** 5872–5876.
2. Nishimura, K., Nakatsu, F., Kashiwagi, K., Ohno, H., Saito, T., and Igarashi, K. (2002) Essential role of *S*-adenosylmethionine decarboxylase in mouse embryonic development. *Genes Cells* **7,** 41–47.
3. Xiong, H., Stanley, B. A., and Pegg, A. E. (1999) Role of Cysteine-82 in the catalytic mechanism of human *S*-adenosylmethionine decarboxylase. *Biochemistry* **38,** 2462–2470.
4. Ekstrom, J. L., Mathews, I. I., Stanley, B. A., Pegg, A. E., and Ealick, S. E. (1999) The crystal structure of human *S*-adenosylmethionine decarboxylase at 2.25 Å resolution reveals a novel fold. *Structure* **7,** 583–595.
5. Toms, A. V., Kinsland, C., McCloskey, D. E., Pegg, A. E., and Ealick, S. E. (2004) Evolutionary links as revealed by the structure of *Thermotoga maritima S*-adenosylmethionine decarboxylase. *J. Biol. Chem.* **279,** 33,837–33,846.
6. Tolbert, W. D., Ekstrom, J. L., Mathews, I. I., et al. (2001) The structural basis for substrate specificity and inhibition of human *S*-adenosylmethionine decarboxylase. *Biochemistry* **40,** 9484–9494.
7. Markham, G. D., Norrby, P., and Bock, C. W. (2002) *S*-adenosylmethionine conformations in solution and in protein complexes: Conformational influences of the sulfonium group. *Biochemistry* **41,** 7636–7646.
8. Stanley, B. A. (1995) Mammalian *S*-adenosylmethionine decarboxylase regulation and processing. In: *Polyamines:Regulation and Molecular Interaction* (Casero, R. A., ed.), RG Landes Co., Austin, TX, pp. 27–75.
9. Pegg, A. E., Xiong, H., Feith, D. J., and Shantz, L. M. (1998) *S*-adenosylmethionine decarboxylase: structure, function and regulation by polyamines. *Biochem. Soc. Trans.* **26,** 580–586.
10. Shantz, L. M. and Pegg, A. E. (1999) Translational regulation of ornithine decarboxylase and other enzymes of the polyamine pathway. *Int. J. Biochem. Cell Biol.* **31,** 107–122.
11. Mari, S. C., Crozat, A., and Jänne, O. A. (1992) Structure and organization of the human *S*-adenosylmethionine decarboxylase gene. *J. Biol. Chem.* **267,** 18,915–18,923.
12. Pajunen, A., Crozat, A., Jännes, O. A., et al. (1988) Structure and regulation of mammalian *S*-adenosylmethionine decarboxylase. *J. Biol. Chem.* **263,** 17,040–17,049.
13. Shantz, L. M., Holm, I., Jänne, O. A., and Pegg, A. E. (1992) Regulation of *S*-adenosylmethionine decarboxylase activity by alterations in the intracellular polyamine content. *Biochem. J.* **288,** 511–518.
14. Hurta, R. A. R. (2000) Altered ornithine decarboxylase and *S*-adenosylmethionine decarboxylase expression and regulation in mouse fibroblasts transformed with oncogenes or constitutively active mitogen-activated protein (MAP) kinase. *Mol. Cell. Biochem.* **215,** 81–92.
15. Hardin, M. S. and Hurta, R. A. (2002) Growth factor-mediated altered expression and regulation of *S*-adenosylmethionine decarboxylase in a H-ras transformed cell line capable of malignant progression. *J. Cell. Biochem.* **84,** 349–358.
16. Bielecki, D. and Hurta, R. A. R. (2000) Insulin-mediated alterations in *S*-adenosylmethionine decarboxylase expression in H-ras transformed cells of varying degrees of malignancy. *Cell. Signal.* **12,** 451–456.

17. Soininen, T., Liisanantti, M. K., and Pajunen, E. I. (1996) *S*-adenosylmethionine decarboxylase gene expression in rat hepatoma cells: Regulation by insulin and by inhibition of protein synthesis. *Biochem. J.* **316,** 273–277.
18. Fredlund, J. O., Johansson, M. C., Dahlberg, E. A., and Oredsson, S. M. (1995) Ornithine decarboxylase and *S*-adenosylmethionine decarboxylase expression during the cell cycle of chinese hamster ovary cells. *Exp. Cell Res.* **216,** 86–92.
19. Kameji, T. and Pegg, A. E. (1987) Inhibition of translation of mRNAs for ornithine decarboxylase and *S*-adenosylmethionine decarboxylase by polyamines. *J. Biol. Chem.* **262,** 2427–2430.
20. White, M. W., Degnin, C., Hill, J., and Morris, D. R. Specific regulation by endogenous polyamines of translational initiation of *S*-adenosylmethionine decarboxylase mRNA in Swiss 3T3 fibroblasts. *Biochem. J.* **268,** 657–660.
21. Kozak, M. (2002) Pushing the limits of the scanning mechanism for initiation of translation. *Gene* **299,** 1–34.
22. Shantz, L. M., Viswanath, R., and Pegg, A. E. (1994) Role of the 5′-untranslated region of mRNA in the synthesis of *S*-adenosylmethionine decarboxylase and its regulation by spermine. *Biochem. J.* **302,** 765–772.
23. Kozak, M. (1991) Structural features in eukaryotic mRNAs that modulate the initiation of translation. *J. Biol. Chem.* **266,** 19,867–19,870.
24. Shantz, L. M. and Pegg, A. E. (1994) Overproduction of ornithine decarboxylase caused by relief of translational repression is associated with neoplastic transformation. *Cancer Res.* **54,** 2313–2316.
25. Hill, J. R. and Morris, D. R. (1992) Cell-specific translation of *S*-adenosylmethionine decarboxylase mRNA. Regulation by the 5′ transcript leader. *J. Biol. Chem.* **267,** 21,886–21,893.
26. Geballe, A. P. and Sachs, M. S. (2000) Translational control by upstream open reading frames. In: *Translational Control of Gene Expression* (Sonenberg, N., Hershey, J. W. B., and Mathews, M. B., eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 595–614.
27. Morris, D. R. and Geballe, A. P. (2000) Upstream open reading frames as regulators of mRNA translation. *Mol. Cell. Biol.* **20,** 8635–8642.
28. Meijer, H. A. and Thomas, A. A. M. (2002) Control of eukaryotic protein synthesis by upstream open reading frames in the 5′-untranslated region of an mRNA. *Biochem. J.* **367,** 1–11.
29. Kozak M. (1999) Initiation of translation in prokaryotes and eukaryotes. *Gene* **234,** 187–208.
30. Ruan, H., Shantz, L. M., Pegg, A. E., and Morris, D. R. (1996) The upstream open reading frame of the mRNA encoding *S*-adenosylmethionine decarboxylase is a polyamine-responsive translational control element. *J. Biol. Chem.* **271,** 29,576–29,582.
31. Hill, J. R. and Morris, D. R. (1993) Cell-specific translational regulation of *S*-adenosylmethionine decarboxylase mRNA. Dependence on translation and coding capacity of the *cis*-acting upstream open reading frame. *J. Biol. Chem.* **268,** 726–731.
32. Mize, G. J., Ruan, H., Low, J. J., and Morris, D. R. (1998) The inhibitory upstream open reading frame from mammalian *S*-adenosylmethionine decarboxylase mRNA has a strict sequence specificity in critical positions. *J. Biol. Chem.* **271,** 32,500–32,505.
33. Mize, G. J. and Morris, D. R. (2001) A mammalian sequence-dependent upstream open reading frame mediates polyamine-regulated translation in yeast. *RNA* **7,** 374–381.
34. Raney, A., Baron, A. C., Mize, G. J., Law, G. L., and Morris, D. R. (2000) *In vitro* translation of the upstream open reading frame in the mammalian mRNA encoding *S*-adenosylmethionine decarboxylase. *J. Biol. Chem.* **275,** 24,444–24,450.

35. Sachs, M. S., Wang, Z., Gaba, A., et al. (2002) Toeprint analysis of the positioning of translation apparatus components at initiation and termination codons of fungal mRNAs. *Methods* **26,** 105–114.
36. Law, G. L., Raney, A., Heusner, C., and Morris, D. R. (2001) Polyamine regulation of ribosome pausing at the upstream open reading frame of *S*-adenosylmethionine decarboxylase. *J. Biol. Chem.* **276,** 38,036–38,043.
37. Welch, E. M., Wang, W., and Peltz, S. W. (2000) Translation termination: It's not the end of the story. In: *Translational Control of Gene Expression* (Sonenberg, N., Hershey, J. W. B., and Mathews, M. B., eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 467–485.
38. Raney, A., Law, G. L., Mize, G. J., and Morris, D. R. (2002) Regulated translation termination at the upstream open reading frame in *S*-adenosylmethionine decarboxylase mRNA. *J. Biol. Chem.* **277,** 5988–5994.
39. Franceschetti, M., Hanfrey, C., Scaramagli, S., et al. (2001) Characterization of monocot and dicot plant *S*-adenosylmethionine decarboxylase gene families including identification in the mRNA of a highly conserved pair of upstream overlapping open reading frames. *Biochem. J.* **353,** 403–409.
40. Hanfrey, C., Franceschetti, M., Mayer, M. J., Illingworth, C., and Michael, A. J. (2002) Abrogation of upstream open reading frame-mediated translational control of a plant *S*-adenosylmethionine decarboxylase results on polyamine disruption and growth perturbations. *J. Biol. Chem.* **277,** 44,131–44,139.
41. Hackert, M. L. and Pegg, A. E. (1997) Pyruvoyl-dependent enzymes. In: *Comprehensive Biological Catalysis, Vol. 2* (Sinnot, M. L., ed.), Academic Press, Orlando, FL, pp. 201–216.
42. Stanley, B. A., Pegg, A. E., and Holm, I. (1989) Site of pyruvate formation and processing of mammalian *S*-adenosylmethionine decarboxylase proenzyme. *J. Biol. Chem.* **264,** 21,073–21,079.
43. Tolbert, W. D., Zhang, Y., Cottet, S. E., et al. (2003) Mechanism of human *S*-adenosylmethionine decarboxylase proenzyme processing as revealed by the structure of the S68A mutant. *Biochemistry* **42,** 2386–2395.
44. Stanley, B. A. and Pegg, A. E. (1991) Amino acid residues necessary for putrescine stimulation of human S-adenosylmethionine decarboxylase proenzyme processing and catalytic activity. *J. Biol. Chem.* **266,** 18,502–18,506.
45. Xiong, H. and Pegg, A. E. (1999) Mechanistic studies of the processing of human *S*-adenosylmethionine decarboxylase proenzyme. *J. Biol. Chem.* **274,** 35,059–35,066.
46. Ekstrom, J. L., Tolbert, W. D., Xiong, H., Pegg, A. E., and Ealick, S. E. (2001) Structure of human *S*-adenosylmethionine decarboxylase self-processing ester intermediate and mechanism of putrescine stimulation of processing as revealed by the H243A mutant. *Biochemistry* **40,** 9495–9504.
47. Kameji, T. and Pegg, A. E. (1987) Effect of putrescine on the synthesis of *S*-adenosylmethionine decarboxylase. *Biochem. J.* **243,** 285–288.
48. Xiong, H., Stanley, B. A., Tekwani, B. L., and Pegg, A. E. (1997) Processing of mammalian and plant *S*-adenosylmethionine decarboxylase proenzymes. *J. Biol. Chem.* **272,** 28,342–28,348.
49. Bennett, E. M., Ekstrom, J. L., Pegg, A. E., and Ealick, S. E. (2002) Monomeric *S*-adenosylmethionine decarboxylase from plants provides an alternative to putrescine stimulation. *Biochemistry* **42,** 14,509–14,517.
50. Stanley, B. A., Shantz, L. M., and Pegg, A. E. (1994) Expression of mammalian *S*-adenosylmethionine decarboxylase in *Escherichia coli*. *J. Biol. Chem.* **269,** 7901–7907.

51. Clyne, T., Kinch, L. N., and Phillips, M. A. (2002) Putrescine activation of *Trypanosoma cruzi S*-adenosylmethionine decarboxylase. *Biochemistry* **41,** 13,207–13,216.
52. Müller, S., Da'dara, A., Lüersen, K., et al. (2000) In the human malaria parasite *Plasmodium falciparum*, polyamines are synthesized by a bifunctional ornithine decarboxylase, *S*-adenosylmethionine decarboxylase. *J. Biol. Chem.* **275,** 8097–8102.
53. Wrenger, C., Lüersen, K., Krause, T., Müller, S., and Walter, R. D. (2001) The *Plasmodium falciparum* bifunctional ornithine decarboxylase, *S*-adenosyl-L-methionine decarboxylase, enables a well balanced polyamine synthesis without domain-domain interaction. *J. Biol. Chem.* **276,** 29,651–29,656.
54. Ndjonka, D., Da'dara, A., Walter, R. D., and Lüersen, K. (2003) *Caenorhabditis elegans S*-adenosylmethionine decarboxylase is highly stimulated by putrescine but exhibits low specificity for activator binding. *Biol. Chem.* **384,** 83–91.
55. Hershko, A. and Ciechanover, A. (1998) The ubiquitin system. *Annu. Rev. Biochem.* **67,** 425–479.
56. Pickart, C. M. (2004) Back to the future with ubiquitin. *Cell* **116,** 181–190.
57. Yerlikaya, A. and Stanley, B. A. (2004) *S*-adenosylmethionine decarboxylase degradation by the 26 S proteasome is accelerated by substrate-mediated transamination. *J. Biol. Chem.* **279,** 12,469–12,478.
58. Murakami, Y., Matsufuji, S., Kameji, T., et al. (1992) Ornithine decarboxylase is degraded by the 26 S proteasome without ubiquitination. *Nature* **360,** 597–599.
59. Berntsson, P. S. H., Alm, K., and Oredsson, S. M. (1999) Half-lives of ornithine decarboxylase and *S*-adenosylmethionine decarboxylase activities during the cell cycle of Chinese hamster ovary cells. *Biochem. Biophys. Res. Commun.* **263,** 13–16.
60. Li, Y., Hess, S., Pannell, L. K., Tabor, C. W., and Tabor, H. (2001) *In vivo* mechanism-based inactivation of *S*-adenosylmethionine decarboxylases from *Escherichia coli*, *Salmonella typhimurium*, and *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **98,** 10,578–10,583.
61. Shantz, L. M., Stanley, B. A., Secrist, J. A., and Pegg, A. E. (1992) Purification of human *S*-adenosylmethionine decarboxylase expressed in *Escherichia coli* and use of this protein to investigate the mechanism of inhibition by the irreversible inhibitors, 5′-deoxy-5′-[(3-hydrazinopropyl)methylamino]adenosine and 5′-([(*Z*)-4-amino-2-butenyl]methylamino)-5′-deoxyadenosine. *Biochemistry* **31,** 6848–6855.
62. Nasizadeh, S. and Persson, L. (2003) Extremely rapid turnover of *S*-adenosylmethionine decarboxylase in *Crithidia fasciculata*. *FEBS Letts.* **553,** 131–134.
63. Hillary, R. A. and Pegg, A. E. (2003) Decarboxylases involved in polyamine biosynthesis and their inactivation by nitric oxide. *Biochim. Biophys. Acta* **1647,** 161–166.
64. Marton, L. J. and Pegg, A. E. (1995) Polyamines as targets for therapeutic intervention. *Annu. Rev. Pharamacol. Toxicol.* **35,** 55–91.
65. Seiler, N. (2003) Thirty years of polyamine-related approaches to cancer therapy. Retrospect and prospect. Part 1. Selective enzyme inhibitors. *Curr. Drug Targets* **7,** 537–564.

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Genetic Engineering of Polyamine Catabolism in Transgenic Mice and Rats

Juhani Jänne, Leena Alhonen, Marko Pietilä, Tuomo A. Keinänen, Suvikki Suppola, Eija Pirinen, Mervi T. Hyvönen, Aki Järvinen, and Markku Laakso

1. Introduction

The biosynthetic pathway of the polyamines is practically irreversible because it involves two decarboxylation reactions. The first evidence, however, indicating that spermidine can be converted to putrescine and spermine to spermidine emerged in the late 1960s when Siimes *(1)* found that radioactive spermidine and spermine yielded labeled putrescine and spermidine in rat liver in vivo. An additional 10 yr were required before the first enzyme, polyamine oxidase (PAO), of a separate polyamine backconversion pathway was purified and characterized *(2)*. Polyamine oxidase strongly favors acetylated spermidine and spermine over the natural polyamines as its substrates *(2)*. That the oxidation of unmodified spermidine and spermine is greatly enhanced by benzaldehyde (or other aldehydes) is in all likelihood attributable to Schiff base formation between the primary amino groups of the polyamine and the aldehydes, thus mimicking the charge distribution of acetylated polyamines *(2)*. It soon became evident that spermidine and spermine are acetylated by a cytosolic enzyme, spermidine/spermine N^1-acetyltransferase (SSAT), that is highly inducible and has a very short half-life *(3)*. Recently, another oxidase involved in polyamine catabolism was found and named spermine oxidase *(4,5)*. The latter enzyme is practically specific for spermine and does not catalyze the oxidation of spermidine or acetylated polyamines *(4,5)*. In the SSAT/PAO-dependent backconversion pathway, SSAT clearly is the rate-controlling enzyme because PAO is a constitutively expressed enzyme occurring in great excess in comparison with inducible SSAT *(6)*. Moreover, PAO oxidizes only acetylated polyamines.

Overexpression of SSAT is sufficient to activate polyamine catabolism in transgenic rodents. We have overexpressed *SSAT* (under its own promoter or heavy metal-inducible

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metallothionein I promoter) in transgenic mice and rats, and likewise generated SSAT-deficient mouse embryonic stem (ES) cells and gene-disrupted mice.

2. Overexpression of SSAT in Transgenic Mice and Rats

An activation of polyamine catabolism through the overexpression of SSAT does not only affect polyamine homeostasis in different transgenic tissues, but also induces a variety of phenotypical changes affecting a number of organs and tissues.

2.1. Effect on Tissue Polyamine Homeostasis

The members of the first established transgenic mouse line overexpressing SSAT harbored 20–50 extra gene copies in their genome. These animals displayed all the changes indicative of activated polyamine catabolism in their tissues. There was a massive accumulation of putrescine, an appearance of N^1-acetylspermidine not normally found in rodent tissues, a distinct decrease in spermidine or spermine pools, and greatly enhanced ornithine decarboxylase (ODC) activity *(7)*. The increase in ODC activity is in all likelihood induced by depleted pools of spermidine and spermine. To normalize the tissue polyamine homeostasis, we generated a hybrid transgenic mouse line overexpressing both ODC and SSAT under the mouse metallothionein I promoter. Unexpectedly, the hybrid animals displayed even more striking signs of accelerated polyamine catabolism than those overexpressing SSAT only *(8)*. In spite of a massive expansion of tissue pools of putrescine, the depleted spermidine and spermine pools were not replenished. This phenomenon can be explained in terms that concurrent overexpression of ODC and SSAT only accelerated the flow of polyamines through "the polyamine cycle." Figure 1 depicts an imaginary polyamine cycle in SSAT-overexpressing transgenic tissues, where both SSAT and ODC activities are elevated. The cycle apparently circles with no net accumulation of spermidine or spermine and the constant supply of putrescine derived from ODC action keeps the cycle running. This scheme is supported by earlier findings with cultured cells indicating that, during intense activation of polyamine catabolism, depleted spermidine and spermine pools cannot be replenished with exogenous polyamines as they are rapidly acetylated, degraded, or excreted *(9)*. A specific inhibition of ODC activity slows down the cycle and distinctly reduces the pool of putrescine. One may also notice that each round consumes four adenosine triphosphate equivalents.

2.2. Regulation of Transgene-Derived SSAT *Gene Expression*

Overexpression of SSAT in transgenic rodents not only offers means to study the phenotypical consequences of activated polyamine catabolism, it also offers opportunities to elucidate the regulation of *SSAT* gene expression. Tissues of transgenic animals overexpressing SSAT typically accumulate large amounts SSAT-specific messenger RNA (mRNA), yet the SSAT activity and immunoreactive protein are mostly only marginally elevated *(7,10)*. This suggests that *SSAT* gene expression is under extremely strict temporal control that is likely to occur at some posttranscriptional level. SSAT is known to be powerfully induced by the higher polyamines and especially by their N-alkylated analogs *(6)*. An immense (up to 40,000-fold) hepatic stimulation of SSAT

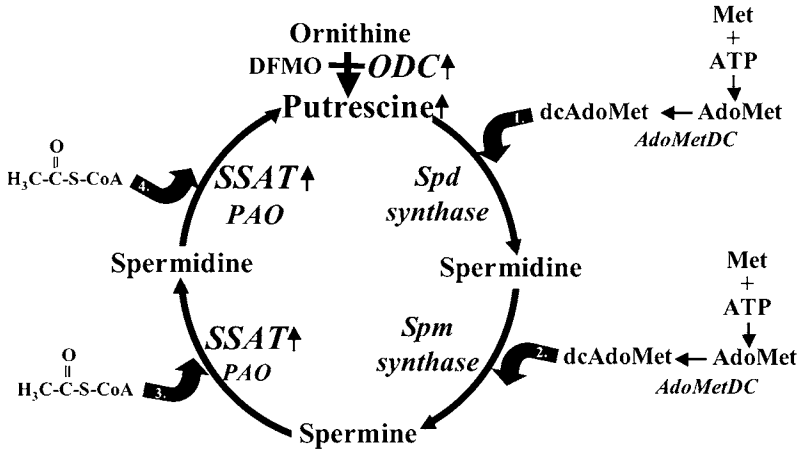

Fig. 1. The polyamine cycle. AdoMetDC, *S*-adenosylmethionine decarboxylase; Spm, spermine; PAO, polyamine oxidase; SSAT, spermidine/spermine N^1-acetyltransferase; ODC, ornithine decarboxylase; DFMO, 2-difluoromethylornithine. The numbers indicate adenosine triphosphate equivalents consumed.

activity was induced by treating transgenic mice overexpressing *SSAT* transgene under the control of mouse metallothionein I promoter with N^1,N^{11}-diethylnorspermine (DENSPM) *(11)*. The latter induction most likely occurred at a level of gene expression downstream of mRNA accumulation, as the transgene was driven by a heterologous promoter not supposed to be induced by polyamine analogs. Some recent evidence indicates that alternative splicing of pre-mRNA may be involved in the regulation of *SSAT* gene expression *(12,13)*. Our unpublished findings have likewise indicated that polyamine analogs influence the splicing of SSAT pre-mRNA. As expected, transgenic mice overexpressing SSAT and fetal fibroblasts derived from them are much more sensitive to the toxic action exerted by polyamine analogs *(10,14)*.

Overexpression of SSAT in transgenic rodents not only disturbs tissue polyamine homeostasis, but also creates a complex phenotype affecting skin, white fat depots, female fertility, liver, pancreas, central nervous system, and the lifespan of the animals.

2.3. Skin

The most striking phenotypical change resulting from SSAT overexpression in transgenic mice is early and permanent hair loss caused by disintegration of hair follicles at the end of the first hair cycle to the formation of follicular cysts *(7,15)*. These follicular cysts are apparently derived from remnants of hair follicles, as indicated by cytokeratin 14 staining *(16)*. The central role of putrescine overaccumulation in these follicular changes was strongly supported by studies indicating that doubly transgenic mice overexpressing both ODC and SSAT with extremely high levels of putrescine in the skin displayed distinctly more severe changes in skin histopathology (significantly larger size of the dermal cysts) than did the singly transgenic mice *(15)*.

During the process of keratinocyte differentiation, proliferating keratinocytes are converted to highly differentiated, nondividing cells. The epidermal keratinocytes express two major pairs of intermediate filaments. One pair, cytokeratin 5 and 14, is specifically expressed in the basal proliferative compartment and the other pair, cytokeratin 1 and 10, is expressed in the differentiating suprabasal compartment *(17)*. SSAT transgenic mouse skin displays an altered differentiation pattern shown as suprabasal cytokeratin 14 expression, reduction of cytokeratin 1 and 10, and a decrease in filaggrin, another differentiation marker. Treatment of the transgenic animals with α-difluoromethylornithine (DFMO), a specific inhibitor of ODC, not only reverted these cutaneous changes, but also induced distinct hair regrowth. The latter drug did not change the levels of skin spermidine or spermine but halved the content of putrescine, again indicating that overaccumulation of putrescine was causally related to the observed skin pathology *(16)*. The mechanism by which elevated putrescine level inhibits keratinocytes differentiation is not known. Putrescine as a small, positively charged molecule may compete with Ca^{2+}, which is essential for keratinocyte differentiation *(17)*. Putrescine may also compete directly with isopeptide formation during cornification of keratinocytes, as it is a natural substrate for transglutaminase *(18)*.

Transgenic mice overexpressing SSAT under its own promoter were more resistant to the two-stage chemical skin carcinogenesis than their wild-type littermates. This resistance may be related to the inability of the tumor promoter to induce ODC activity in the transgenic mice *(15)*. Opposite results were obtained when SSAT expression was directed to the outer root sheath keratinocytes with the aid of the keratin 6 promoter *(19)*. Unlike the animals with systemic SSAT overexpression, these K6-SSAT mice showed higher ODC activities in papillomas than their syngenic littermates *(19)*.

Interestingly, a rare X chromosome-linked syndrome called KFSD (i.e., keratosis follicularis spinulosa decalvans) affecting the skin and eyes may also be related to enhanced polyamine turnover. The syndrome apparently involves a duplication of a region on the X chromosome containing the *SSAT* gene. The skin symptoms of the affected individuals resemble those found in SSAT-overexpressing mice. Analyses of cultured fibroblasts from an affected individual indeed showed signs of activated polyamine catabolism, such as increased SSAT activity, enhanced putrescine accumulation, and a decrease in cellular spermidine pool *(20)*.

2.4. White Fat Depots

The initial characterization of SSAT-overexpressing mice revealed, in addition to hairlessness, practically a total lack of subcutaneous fat depots *(7)*. Subsequently, we found that these animals also lacked visceral fat depots and thus were severely lipoatrophic. In contrast to other lipoatrophic mouse models, SSAT transgenics did not show any triglyceride accumulation in nonadipose tissues and they fully retained insulin sensitivity *(21)*. On fasting, the transgenic animals showed severely impaired ketogenesis, very low plasma leptin level, and significantly reduced triglyceride, glucose, and insulin levels. The transgenic animals also exhibited significantly elevated basal metabolic rate in comparison with the wild-type animals, indicating a greater expenditure of energy *(21)*. It thus appears that generalized SSAT overexpression causes lipoatrophy, but also

protects the animals from the accumulation of fats in nonadipose tissues, insulin resistance, and lipoatrophic diabetes.

2.5. Female Fertility

During the primary characterization of the first SSAT transgenic mouse line, we soon found that the female members of the line were infertile. The apparent reason for the infertility was uterine hypoplasia and the absence of corpora lutea in the ovary *(7)*. The female reproductive organs (uterus and ovary) of transgenic mice likewise displayed slightly different gene expression pattern in comparison with the wild-type animals, namely the expression of lipoprotein lipase and glyceraldehyde-3-phosphate dehydrogenase was elevated in the transgenic females *(22)*. It is, however, entirely unknown whether these changes contribute to uterine hypoplasia and ovarian hypofunction.

2.6. Liver

SSAT-overexpressing mice are extremely sensitive to the toxic effects exerted by polyamine analogs, such as DENSPM, in comparison with their wild-type littermates *(14)*. Although the exact mechanisms of this toxicity are not known, treatment of transgenic mice overexpressing SSAT under the control of metallothionein promoter with DENSPM caused a profound depletion of hepatic spermidine and spermine associated with swelling of hepatic mitochondria and marked mortality in comparison with their wild-type littermates *(11)*. However, our unpublished experiments have shown that SSAT-overexpressing mice are more resistant to general hepatotoxins, such as carbon tetrachloride and thioacetamide, than wild-type animals.

An enhanced biosynthesis of the polyamines is closely associated with early rat liver regeneration after partial hepatectomy *(23)*. Pharmacological intervention of polyamine biosynthesis (inhibition of ODC) during rat liver regeneration has yielded conflicting results *(24,25)*, thus leaving it entirely open whether increased supply of the polyamines is really needed for liver regeneration to occur. When transgenic rats expressing SSAT under the control of the metallothionein promoter were subjected to partial hepatectomy, SSAT activity was dramatically stimulated at 24 h postoperatively, leading to profound depletion of hepatic spermidine and spermine *(26)*. This SSAT induction is apparently related to the known striking induction of hepatic metallothionein expression after partial hepatectomy *(27)*. The near total depletion of the higher polyamines in transgenic livers apparently prevented the initiation of liver regeneration as judged by a variety of proliferation indicators, such as thymidine incorporation, immunohistochemical staining of proliferating cell nuclear antigen, and the increase in weight of the liver remnant. Because of very high ODC activity, the hepatic spermidine pool returned to the preoperative level at 72 h after the operation, followed by a slow commencement of liver regeneration in the transgenic rats *(26)*. The view that the delayed initiation of liver regeneration in the transgenic rats was from polyamine depletion was strongly supported by experiments revealing that a prior administration of 1-methylspermidine, a nontoxic derivative that is not a substrate for SSAT and is supposed to fulfill the cellular functions of the natural polyamine *(28,29)*, fully restored

liver regeneration in the transgenic animals *(30)*. Much of the earlier experimental data appear to indicate that spermidine has a central role in rat liver regeneration. This view is supported by the kinetics of polyamine accumulation in regenerating liver (i.e., there is an early accumulation of putrescine that is apparently rapidly converted to increased levels of spermidine while spermine content, if anything, decreases during the first days of regeneration) *(23)*. Putrescine thus would serve as a precursor for spermidine, and spermine would represent kind of a storage compound to be converted to spermidine when needed. The picture is also complicated by the fact that spermidine has a specific function in eukaryotic cells being converted to deoxyhypusine and further to hypusine, which is an integral component of eukaryotic initiation factor 5A *(31)*. Because the latter factor is essential to mammalian cell proliferation *(32)*, it impossible to judge whether spermidine depletion-associated growth arrest is directly attributable to the polyamine or is a sign of hypusine depletion and hence the reduction of functional initiation factor 5A *(29)*. The restoration of liver regeneration by methylspermidine cannot be taken as proof that the regeneration-promoting effect was attributable to spermidine because this analog likewise can be converted to hypusine *(29)*.

We subsequently carried out experiments with these transgenic rats using another polyamine analog, 1,12-dimethylspermine *(33)*. This compound does not serve as a precursor for hypusine synthesis *(29)*. Dimethylspermine is not entirely stable because it can be converted to methylspermidine (by oxidation) to some extent in vivo *(33)* and can thus be converted to hypusine. We found that liver regeneration in the transgenic rats could be restored also by dimethylspermine under conditions in which the conversion of the latter compound to methylspermidine was insufficient to yield enough methylspermidine for the correction of polyamine depletion and for the initiation of liver regeneration *(33)*. Dimethylspermine also fully reversed DFMO-induced cytostasis under conditions (in the presence of an inhibitor of PAO and spermine oxidase) in which no conversion to methylspermidine occurred. These results can be understood in terms that spermidine and spermine are fully exchangeable in promoting growth under the condition of severe depletion of the natural polyamines and that this effect is independent of hypusine depletion. The growth inhibition attributable to hypusine depletion in all likelihood occurs much later *(34)*.

2.7. Pancreas

In terms of its polyamine content, the pancreas is a highly unique mammalian organ, being the richest source of spermidine and displaying the highest molar ratio (about 10) of spermidine to spermine *(35,36)*. The physiological importance of the very high spermidine content in the pancreas is not known, but it may be related to the intense protein synthesis going on in this organ or to maintenance of pancreatic structural integrity. Relatively little is known about the role of the polyamines in pancreatic growth and function. Although DFMO retards the growth of the pancreas *(37)*, it apparently does not disturb the secretory function of the exocrine pancreas *(38)*. No experimental findings exist indicating an involvement of the polyamines in the development of pancreatitis. The transgenic rats with heavy metal-inducible metallothionein promotor-driven *SSAT* offered a meaningful opportunity to study the roles of the higher polyamines in

the pancreas. Metallothionein I is predominantly expressed in the pancreas and liver (and to some extent in the kidney) and it is powerfully induced by heavy metals, such as zinc. In the absence of any induction, these transgenic rats showed distinct signs of the activation of polyamine catabolism in the pancreas, such as greatly enhanced accumulation of putrescine and the appearance of N^1-acetylspermidine, yet the pools of higher polyamines were relatively well preserved in comparison with the wild-type animals *(39)*. Nontoxic doses of zinc powerfully induced the promoter and resulted in a striking stimulation of pancreatic SSAT that led to an almost total depletion of pancreatic spermidine and spermine pools and massive accumulation of putrescine *(39)*. No such changes were found in wild-type animals similarly exposed to zinc. In the transgenic animals, the activation of polyamine catabolism by zinc was accompanied by a rapid development (within 24 h) of acute necrotizing pancreatitis, as verified by histology and elevated plasma α-amylase activity *(39)*. The oxidation of the higher polyamines by PAO generates, in addition to putrescine and spermidine, hydrogen peroxide and acetaminopropanol, which are extremely reactive and could contribute to the development of the pancreatitis. The latter possibility was excluded by experiments in which the animals were treated with zinc combined with a PAO inhibitor. In terms of the severity of pancreatitis, the combined regimen, if anything, made the situation even worse, thus excluding the contribution of reactive oxygen species to the development of the organ inflammation *(39)*.

The view that the development of pancreatitis in these transgenic animals on induction of SSAT was causally related to the profound depletion of pancreatic spermidine and spermine was strongly supported, even proven, by experimental results indicating that a prior administration of the spermidine analog, 1-methylspermidine, totally prevented the zinc-induced pancreatitis, as verified by histology and plasma α-amylase activity *(30)*. The development of pancreatitis in response to SSAT induction is not confined to transgenic rats because transgenic mice harboring metallothionein promoter-driven *SSAT* transgene similarly develop acute pancreatitis on exposure to zinc *(40)*.

Although the exact cellular functions of pancreatic polyamines remain to be elucidated, the preservation of sufficient pools of spermidine and spermine appears to be required for the maintenance of functional and structural integrity of the pancreas. Moreover, our preliminary experiments have revealed that an activation of polyamine catabolism is a universal phenomenon involved in practically all experimental animal models of acute pancreatitis. The created transgenic model of pancreatitis, which is highly similar to human pancreatitis, may offer an excellent and sensitive means for screening drugs potentially able to induce pancreatitis. A recent example is gossypol, a cotton seed-derived male antifertility agent *(41)*, which activates pancreatic polyamine catabolism and induces acute pancreatitis in transgenic rats overexpressing SSAT *(42)*.

2.8. Central Nervous System

There seems to be a continuous debate concerning the role of altered polyamine metabolism in insults affecting the central nervous system. Enhanced accumulation of putrescine, as resulted from ODC induction, is more often considered a cause of neuronal damage than a sign of plasticity and neuroprotection *(43)*. An induction of SSAT

is likewise linked to the development of neuronal damage because the neurotoxin kainate-induced seizure activity was associated with enhanced SSAT activity *(44)*. However, our experiments with transgenic mice overexpressing SSAT yielded just the opposite results, indicating that these animals in comparison with their wild-type littermates are protected from kainate-induced toxicity. This was clearly manifested as greatly reduced (by 50%) overall mortality and substantially diminished neuronal damage and loss of neurons *(45)*. The neuroprotection by the transgenicity is in all likelihood attributable to the greatly expanded putrescine pools in a number of different brain regions. In an analogous fashion, these transgenic mice showed an elevated threshold to pentylenetetrazol-induced tonic and clonic convulsions in comparison with their wild-type littermates *(46)*. This difference between the genotypes, however, disappeared when the convulsant was administered together with a *N*-methyl-D-aspartate (NMDA) receptor antagonist *(46)*.

The SSAT transgenic mice have also been subjected to neurobehavioral profiling assessment, which revealed that these animals are hypomotoric, less aggressive, and have spatial learning impairment in comparison with wild-type mice *(47)*.

Taken together, these results led us to conclude that the dramatically elevated putrescine content and the up to 40-fold increased molar ratio of putrescine to the higher polyamines in the brain of SSAT-overexpressing mice create a partial blockade of the NMDA receptor, at which spermidine and spermine act as agonists *(48)*.

2.9. Life Span

Hybrid transgenic mice concurrently overexpressing both ODC and SSAT have greatly reduced life span in comparison with wild-type animals *(8)*.

Transgenic mice overexpressing SSAT either under its own promoter or under mouse metallothionein I promoter were observed for changes in life span. An overexpression of SSAT, irrespective of the promoter, led to a substantial reduction in their life span. In fact, all the transgenic mice died at the age of about 1 yr, whereas 90% of the wild-type mice were still alive at that time. Even though the reasons for this shortened life span are not known, it is highly probable that some sort of oxidative stress created by the constitutively enhanced oxidation of the higher polyamines contribute to the early death of the transgenic animals. Under these conditions, the "polyamine cycle" (Fig. 1) supposedly goes to great overdrive.

3. Targeted Disruption of *SSAT* Gene

3.1. Targeted Disruption of SSAT Gene in ES Cells

Targeted disruption of the *SSAT* gene has surprisingly little effect on the general polyamine homeostasis in mouse ES cells with the possible exception of constantly elevated spermidine pool. The growth characteristics of the mutated cells also were indistinguishable from those of the parental cells *(49)*. Although the SSAT-deficient cells were more resistant, as expected, to the growth-inhibitory action of polyamine analogs, the contribution of SSAT deficiency to the observed resistance is somewhat confusing, as the polyamine analog equally and effectively depleted the polyamines in the wild-type and mutated cells *(49)*. These results can be understood in terms that

direct replacement of the natural polyamine from their intracellular binding sites by the analogs, rather than their enhanced oxidation or excretion of acetylated polyamines, is the major mode of the action of the analogs. The situation may be entirely different in cells overexpressing SSAT *(10)*.

Nevertheless, tracer experiments with the SSAT-deficient mouse ES cells generated the important finding proving that SSAT activity is absolutely required for the conversion of spermidine to putrescine, but not at all for the formation of spermidine from spermine, thus being in line with the existence of a separate spermine oxidase *(49)*.

3.2. Targeted Disruption of SSAT *Gene in Mouse*

We have recently generated SSAT-deficient mice from the ES cells, the phenotypical characterization of which is under way. Preliminary analyses indicate that polyamine homeostasis is well preserved in the knockout mice as well.

4. Conclusions

In terms of polyamine metabolism, it is clear that a transgenic activation of polyamine catabolism through an overexpression of SSAT is a much more powerful tool to unbalance tissue polyamine homeostasis than a transgenic activation of their biosynthesis. Although an overexpression of ODC in transgenic rodents brings about many interesting phenotypical features, such as male infertility, hairlessness, enhanced skin carcinogenesis, and apparent neuroprotection, the inherent limitation of these experiments is the fact that ODC overexpression only enhances the accumulation of tissue putrescine, which is apparently not converted to higher polyamines *(48)*. However, the experimental work with transgenic animals has certainly created new information of the putative cellular roles of putrescine. Overaccumulation of putrescine in the skin, whether derived from enhanced ODC or SSAT activity, leads to early loss of hair, apparently because of disturbed keratinocyte differentiation *(15,48)*. The view that overaccumulated putrescine in the skin is responsible for the loss of hair is strongly supported by the findings indicating that a reduction of skin putrescine by DFMO induces hair regrowth in transgenic animals overexpressing either SSAT or ODC *(16,50)*. In a similar fashion, all the experimental data obtained with transgenic animals overaccumulating putrescine in the brain as a result of ODC or SSAT overexpression appear to indicate that an enhanced formation of putrescine in response to brain insults is a neuroprotective measure rather than a cause of neuronal damage *(48)*. A plausible mechanistic explanation for this phenomenon would be a partial blockade of the NMDA receptor by greatly elevated levels of putrescine.

Some of the experimental findings obtained from the SSAT-overexpressing rats appear to assign specific growth-promoting roles for the higher polyamines. This is especially true for rat liver regeneration in which the higher polyamines, but apparently not putrescine, seem to play a specific role in the initiation of the regenerative process. The experiments with surrogate analogs may indicate that spermidine and spermine are fully exchangeable in this respect. The same experiments likewise suggest that the early polyamine depletion-induced growth inhibition is independent of reduced hypusine formation. What has been said about the liver holds principally true also for the

pancreas. Spermidine or spermine, but again not putrescine, appear to be indispensable in terms of the maintenance of pancreatic integrity, as profound polyamine depletion leads to the development of acute pancreatitis. Again, experiments with the surrogate analogs strongly support the notion that organ inflammation is caused by polyamine depletion *per se*, and not by oxidative stress, for instance.

However, many puzzling questions still remain. The lipoatrophy associated with SSAT overexpression has no immediate explanation. Probably a more important question is why this lipoatrophy in the SSAT transgenics does not lead to insulin resistance and lipoatrophic diabetes. The answer to this question may have therapeutic implications in type 2 diabetes.

It is hoped that the reader will appreciate the power of transgene technology that apparently can yield experimental results not obtainable by more conventional techniques.

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References

1. Siimes, M. (1967) Studies on the metabolism of 1,4-^{14}C-spermidine and 1,4-^{14}C-spermine in the rat. *Acta Physiol. Scand. Suppl.* **298,** 1–66.
2. Hölttä, E. (1997) Oxidation of spermidine and spermine in rat liver: Purification and properties of polyamine oxidase. *Biochemistry* **16,** 91–100.
3. Matsui, I., Wiegand, L., and Pegg, A. E. (1981) Properties of spermidine N-acetyltransferase from livers of rats treated with carbon tetrachloride and its role in the conversion of spermidine into putrescine. *J. Biol. Chem.* **256,** 2454–2458.
4. Wang, Y., Devereux, W., Woster, P. M., et al. (2001) Cloning and characterization of a human polyamine oxidase that is inducible by polyamine analogue exposure. *Cancer Res.* **61,** 5370–5373.
5. Vujcic, S., Diegelman, P., Bacchi, C. J., Kramer, D. L., and Porter, C. W. (2002) Identification and characterization of a novel flavin-containing spermine oxidase of mammalian cell origin. *Biochem. J.* **367,** 665–675.
6. Casero, R. A. and Pegg, A. E. (1993) Spermidine/spermine N1-acetyltransferase—the turning point in polyamine metabolism. *FASEB J.* **7,** 653–661.
7. Pietilä, M., Alhonen, L., Halmekytö, M., Kanter, P., Jänne, J., and Porter, C. W. (1997) Activation of polyamine catabolism profoundly alters tissue polyamine pools and affects hair growth and female fertility in transgenic mice overexpressing spermidine/spermine N1-acetyltransferase. *J. Biol. Chem.* **272,** 18,746–18,751.
8. Suppola, S., Heikkinen, S., Parkkinen, J. J., et al. (2001) Concurrent overexpression of ornithine decarboxylase and spermidine/spermine N1-acetyltransferase further accelerates the catabolism of hepatic polyamines in transgenic mice. *Biochem. J.* **358,** 343–348.
9. Vujcic, S., Halmekytö, M., Diegelman, P., et al. (2000) Effects of conditional overexpression of spermidine/spermine N1-acetyltransferase on polyamine pool dynamics, cell growth, and sensitivity to polyamine analogs. *J. Biol. Chem.* **275,** 38,319–38,328.
10. Alhonen, L., Karppinen, A., Uusi-Oukari, M., et al. (1998) Correlation of polyamine and growth responses to N^1,N^{11}-diethylnorspermine in primary fetal fibroblasts derived from transgenic mice overexpressing spermidine/spermine N^1-acetyltransferase. *J. Biol. Chem.* **273,** 1964–1969.

11. Suppola, S., Pietilä, M., Parkkinen, J. J., et al. (1999) Overexpression of spermidine/spermine N1-acetyltransferase under the control of mouse metallothionein I promoter in transgenic mice: Evidence for a striking post-transcriptional regulation of transgene expression by a polyamine analogue. *Biochem J.* **338,** 311–316.
12. Nikiforova, N. N., Velikodvorskaja, T. V., Kachko, A. V., et al. (2002) Induction of alternatively spliced spermidine/spermine N1-acetyltransferase mRNA in the human kidney cells infected by venezuelan equine encephalitis and tick-borne encephalitis viruses. *Virology* **297,** 163–171.
13. Mita, K., Fukuchi, K., Hamana, K., Ichimura, S., and Nenoi, M. (2004) Accumulation of spermidine/spermine N1-acetyltransferase and alternatively spliced mRNAs as a delayed response of HeLa S3 cells following X-ray irradiation. *Int. J. Radiat. Biol.* **80,** 369–375.
14. Alhonen, L., Pietilä, M., Halmekytö, M., Kramer, D. L., Jänne, J., and Porter, C. W. (1999) Transgenic mice with activated polyamine catabolism due to overexpression of spermidine/spermine N1-acetyltransferase show enhanced sensitivity to the polyamine analog, N1, N11- diethylnorspermine. *Mol. Pharmacol.* **55,** 693–698.
15. Pietilä, M., Parkkinen, J. J., Alhonen, L., and Jänne, J. (2001) Relation of skin polyamines to the hairless phenotype in transgenic mice overexpressing spermidine/spermine N1-acetyltransferase. *J. Invest. Dermatol.* **116,** 801–805.
16. Pietilä, M., Pirinen, E., Keskitalo, S., et al. (2005) Disturbed keratinocyte differentiation in transgenic mice and organotypic keratinocyte cultures as a result of spermidine/spermine N1-acetyltransferase overexpression. *J. Invest. Dermatol.* **124,** 596–601.
17. Hennings, H., Michael, D., Cheng, C., Steinert, P., Holbrook, K., and Yuspa, S. H. (1980) Calcium regulation of growth and differentiation of mouse epidermal cells in culture. *Cell* **19,** 245–254.
18. Piacentini, M., Martinet, N., Beninati, S., and Folk, J. E. (1998) Free and protein-conjugated polyamines in mouse epidermal cells: Effect of high calcium and retinoic acid. *J. Biol. Chem.* **263,** 3790–3794.
19. Coleman, C. S., Pegg, A. E., Megosh, L. C., Guo, Y., Sawicki, J. A., and O'Brien, T. G. (2002) Targeted expression of spermidine/spermine N1-acetyltransferase increases susceptibility to chemically induced skin carcinogenesis. *Carcinogenesis* **23,** 359–364.
20. Gimelli, G., Giglio, S., Zuffardi, O., et al. (2002) Gene dosage of the spermidine/spermine N(1)-acetyltransferase (SSAT) gene with putrescine accumulation in a patient with a Xp21.1p22.12 duplication and keratosis follicularis spinulosa decalvans (KFSD). *Hum. Genet.* **111,** 235–241.
21. Pirinen, E., Heikkinen, S., Virkamäki, A., et al. (2002) Severely reduced white fat deposits without a defect in insulin sensitivity in transgenic mice overexpressing spermidine/spermine N1-acetyltransferase. *Diabetologia* **45,** A83.
22. Min, S. H., Simmen, R. C., Alhonen, L., et al. (2002) Altered levels of growth-related and novel gene transcripts in reproductive and other tissues of female mice over-expressing spermidine/spermine N1-acetyltransferase (SSAT). *J. Biol. Chem.* **277,** 3647–3657.
23. Jänne, J. (1967) Studies on the biosynthetic pathway of polyamines in rat liver. *Acta Physiol. Scand. Suppl.* **300,** 1–71.
24. Harik, S. I., Hollenberg, M. D., and Snyder, S. H. (1974) Alpha-Hydrazino-ornithine blocks net synthesis of putrescine but not of RNA and DNA. *Nature* 1**249,** 250–251.
25. Pösö, H. and Pegg, A. E. (1982) Effect of alpha-difluoromethylornithine on polyamine and DNA synthesis in regenerating rat liver. *Biochim. Biophys. Acta* **696,** 179–186.
26. Alhonen, L., Räsänen, T. L., Sinervirta, R., et al. (2002) Polyamines are required for the initiation of rat liver regeneration. *Biochem. J.* **362,** 149–153.

27. Tsujikawa, K., Suzuki, N., Sagawa, K., et al. (1994) Induction and subcellular localization of metallothionein in regenerating rat liver. *Eur. J. Cell. Biol.* **63,** 240–246.
28. Varnado, B. L., Voci, C. J., Meyer, L. M., and Coward, J. K. (2002) Circular dichroism and NMR studies of metabolically stable alpha-methylpolyamines: Spectral comparison with naturally occurring polyamines. *Bioorg. Chem.* **28,** 395–408.
29. Byers, T. L., Lakanen, J. R., Coward, J. K., and Pegg, A. E. (1994) The role of hypusine depletion in cytostasis induced by S-adenosyl-L-methionine decarboxylase inhibition: New evidence provided by 1-methylspermidine and 1,12-dimethylspermine. *Biochem J.* **303,** 363–368.
30. Räsänen, T. L., Alhonen, L., Sinervirta, R., et al. (2002) A polyamine analogue prevents acute pancreatitis and restores early liver regeneration in transgenic rats with activated polyamine catabolism. *J. Biol. Chem.* **277,** 39,867–39,872.
31. Park, M. H., Cooper, H. L., and Folk, J. E. (1981) Identification of hypusine, an unusual amino acid, in protein from human lymphocytes and of spermidine as its biosynthetic precursor. *Proc. Natl. Acad. Sci. USA* **78,** 2869–2873.
32. Park, M. H., Wolff, E. C., and Folk, J. E. (1993) Is hypusine essential for eukaryotic cell proliferation? *Trends Biochem. Sci.* **18,** 475–479.
33. Järvinen, A., Grigorenko, N., Khomutov, A. R., et al. (2005) Metabolic stability of α-methylated polyamines and their use as substitutes for the natural polyamines. *J. Biol. Chem.* **280,** 6595–6601.
34. Bergeron, R. J., Weimar, W. R., Muller, R., et al. (1998) Effect of polyamine analogues on hypusine content in JURKAT T-cells. *J. Med. Chem.* **41,** 3901–3908.
35. Rosenthal, S. M. and Tabor, C. W. (1956) The pharmacology of spermine and spermidine. Distribution and excretion. *J. Pharmacol. Exp. Ther.* **116,** 131–138.
36. Löser, C., Fölsch, U. R., Cleffmann, U., Nustede, R., and Creuzfeldt, W. (1989) Role of ornithine decarboxylase and polyamines in camostate (Foy-305)-induced pancreatic growth in rats. *Digestion* **43,** 98–112.
37. Morisset, J. and Grondin, G. (1987) Implication of ornithine decarboxylase and polyamines in pancreatic growth of neonatal rats. *Pancreas* **2,** 303–311.
38. Haarstad, H., Skei, T., and Petersen, H. (1989) Inhibition of polyamine synthesis by α-difluoromethyl ornithine and its effect on pancreatic secretion and growth in the rat. *Scand. J. Gastroenterol.* **24,** 733–744.
39. Alhonen, L., Parkkinen, J. J., Keinänen, T., Sinervirta, R., Herzig, K. H., and Jänne, J. (2000) Activation of polyamine catabolism in transgenic rats induces acute pancreatitis. *Proc. Natl. Acad. Sci. USA* **97,** 8290–8295.
40. Herzig, K. H., Jänne, J., and Alhonen, L. (2005) Acute pancreatitis induced by activation of the polyamine catabolism in gene-modified mice and rats overexpressing spermidine/spermine N1-acetyltransferase. *Scand. J. Gastroenterol.* **40,** 120–121.
41. Qian, S. Z. and Wang, Z. G. (1984) Gossypol: A potential antifertility agent for males. *Annu. Rev. Pharmacol. Toxicol.* **24,** 329–360.
42. Räsänen, T. L., Alhonen, L., Sinervirta, R., et al. (2003) Gossypol activates pancreatic polyamine catabolism in normal rats and induces acute pancreatitis in transgenic rats overexpressing spermidine/spermine N1-acetyltransferase. *Scand. J. Gastroenterol.* **38,** 787–793.
43. Kauppinen, R. A. and Alhonen, L. I. (1995) Transgenic animals as models in the study of the neurobiological role of polyamines. *Progr. Neurobiol.* **47,** 545–563.
44. Najm, I., El-Skaf, G., Tocco, G., Vanderklish, P., Lynch, G., and Baudry, M. (1992) Seizure activity-induced changes in polyamine metabolism and neuronal pathology during the postnatal period in rat brain. *Dev. Brain Res.* **69,** 11–21.

45. Kaasinen, K., Koistinaho, J., Alhonen, L., and Jänne, J. (2000) Overexpression of spermidine/spermine N^1-acetyltransferase in transgenic mice protects the animals from kainate-induced toxicity. *Eur. J. Neurosci.* **12,** 540–548.
46. Kaasinen, S. K., Gröhn, O. H., Keinänen, T. A., Alhonen, L., and Jänne, J. (2003) Overexpression of spermidine/spermine N1-acetyltransferase elevates the threshold to pentylenetetrazol-induced seizure activity in transgenic mice. *Exp. Neurol.* **183,** 645–652.
47. Kaasinen, S. K., Oksman, M., Alhonen, L., Tanila, H., and Jänne, J. (2004) Spermidine/spermine N1-acetyltransferase overexpression in mice induces hypoactivity and spatial learning impairment. *Pharmacol. Biochem. Behav.* **78,** 35–45.
48. Jänne, J., Alhonen, L., Pietilä, M., and Keinänen, T. A. (2004) Genetic approaches to the cellular functions of polyamines in mammals. *Eur. J. Biochem.* **271,** 877–894.
49. Niiranen, K., Pietilä, M., Pirttilä, T. J., et al. (2002) Targeted disruption of spermidine/spermine N1-acetyltransferase gene in mouse embryonic stem cells. Effects on polyamine homeostasis and sensitivity to polyamine analogues. *J. Biol. Chem.* **277,** 25,323–25,328.
50. Soler, A. P., Gilliard, G., Megosh, L. C., and O'Brien, T. G. (1996) Modulation of murine hair follicle function by alterations in ornithine decarboxylase activity. *J. Invest. Dermatol.* **106,** 1108–1113.

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