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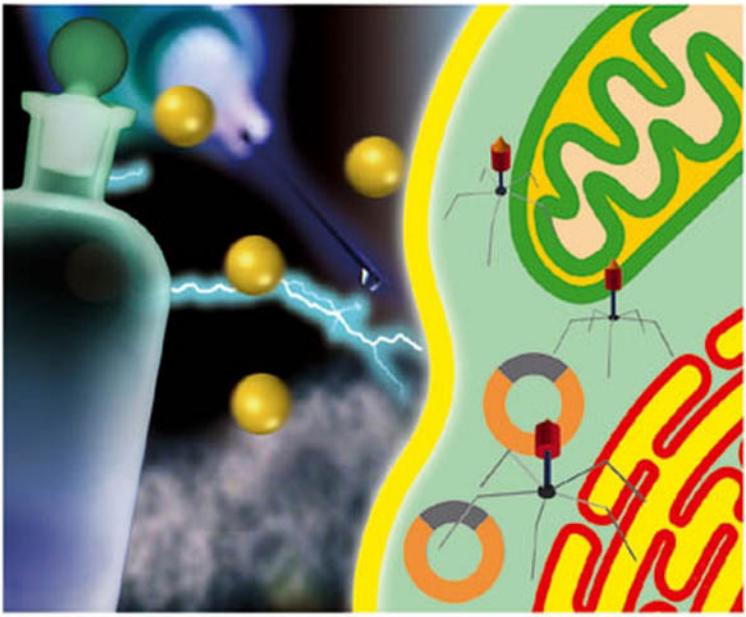
DNA-Pharmaceuticals

Formulation and Delivery in Gene Therapy, DNA Vaccination and Immunotherapy



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Martin Schleef

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Editor

Dr. Martin Schleef
PlasmidFactory GmbH & Co. KG
Meisenstrasse 96
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Germany
www.plasmidfactory.com

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Preface

The difference between "pharmaceuticals" and "modern" or „innovative pharmaceuticals" like nucleic acids (e.g. plasmids, DNA fragments, RNA, viruses or virus-like-particles) is more or less open to interpretation of those developing these to improve safety, functionality, stability or economic aspects (in production and marketing). However, no doubt exists on the existence of a completely new class of active pharmaceutical ingredients (API) when the use of such genetic material for a preventive or curative application was discovered. On one side the need for new products with respect to patent situation and marketing is eminent and on the other side safety concerns for patient and environment are discussed. Furthermore questions like "why changing to a new type of product if the old one still works" are not rare and need to be addressed on the level of market supply costs (were DNA is not expensive) rather than comparing dose costs for existing pharmaceuticals with those for pre-clinical or phase I and II clinical material.

Earlier (in Schleef: "Plasmids for therapy and vaccination", Wiley-VCH 2001) we presented the vector type and clinical approaches of plasmid vectors. This new book extends those subjects into the next step after design and manufacturing of plasmid DNA pharmaceuticals: The focus is on the route of administration, quality control and regulatory aspects.

After a short overview on DNA vaccination (Chapter 1) and a comprehensive summary of regulatory aspects for this class of pharmaceuticals (Chapter 2), the new aspects of improving functionality (e.g. targeting) and purity (ccc-form of plasmid DNA vs. other topologies and contaminants as well as production technology; Chapter 3) or minimizing the vector system (Chapter 4; further progress is expected shortly) are presented.

A special overview on formulation and delivery is presented with Chapters 5 and 6 is a successful example for large animal veterinary DNA vaccine development.

Chapters 6 to 16 indicate the important (different) ways of introducing the vector to the tissue (and cell compartment) of interest. Due to a recently increased interest in electro gene transfer we decided to have two chapters (Chapters 11 and 12) on this subject included. The use of plasmid based siRNA technology was found to be of interest and an example is presented within Chapter 13.

We are aware of the fact that these 13 chapters only represent a small part of the ongoing development in this highly dynamic field. The economic and social relevance of the innovative class of these pharmaceuticals is clearly visible.

For all those who like to further discuss these aspects I look forward to do so at any time (martin.schleef@plasmidfactor.com). My thank is directed to all authors and co-authors of this book and all others making it possible.

Special thanks go to all volunteers of clinical trials with DNA pharmaceuticals.

Bielefeld, August 2005 *Martin Schleef*

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List of Contributors

Vitali Alexeev
Department of Dermatology and
Cutaneous Biology
Jefferson Medical College
233 South 10th Street
Suite 326 BLSB
Philadelphia, PA 19107
USA

Pascal Bigey
Laboratoire de Pharmacologie
Chimique et Génétique
INSERM U640 – CNRS UMR8151
Université René Descartes – ENSCP
Faculté de Pharmacie
4, Avenue de l'Observatoire
75270 Paris Cédex 06
France

Brian Bigger
Stem Cell Research Group
John Radcliffe Hospital
University of Oxford
Oxford OX3 9BQ
United Kingdom

Charles Coutelle
Gene Therapy Research Group
Imperial College
Exhibition Road
London SW7 2AZ
United Kingdom

Lee A. Davies
Gene Medicine Group
John Radcliffe Hospital
University of Oxford
Oxford OX3 9DU
United Kingdom

John Fabre
Department of Clinical Science
The Rayne Institute
Guy's, King's and St. Thomas' School
of Medicine
123 Coldharbour Lane
London SE5 9NU
United Kingdom

Matthias Giese
Im Schaffner 24
69123 Heidelberg
Germany

Deborah Gill
Gene Medicine Group
John Radcliffe Hospital
University of Oxford
Oxford OX3 9DU
United Kingdom

Richard Harbottle
Gene Therapy Research Group
Imperial College
Exhibition Road
London SW7 2AZ
United Kingdom

Stephen C. Hyde
Gene Medicine Group
John Radcliffe Hospital
University of Oxford
Oxford OX3 9DU
United Kingdom

Carsten Kneuer
Institute for Pharmacology,
Pharmacy and Toxicology
University of Leipzig
An den Tierkliniken 15
04103 Leipzig
Germany

Margaret Liu
3656 Happy Valley Road
Lafayette, CA 94549
USA
and
11, Rue de Molsheim
67082 Strasbourg
France

Lluis M. Mir
Laboratory of Vectorology and
Gene Transfer
UMR 8121 CNRS –
Institut Gustave-Roussy
39, Rue C. Desmoulins
94805 Villejuif Cédex
France

Christian Plank
Department for Experimental
Oncology (IEOT)
Munich Technical University
Ismaninger Strasse 22
81675 Munich
Germany

Carsten Rudolph
Department of Molecular Pneumology
Dr. von Hauner Children's Hospital
Ludwig-Maximilians-University
Lindwurmstrasse 4
80337 Munich
Germany

Franz Scherer
Department for Experimental
Oncology (IEOT)
Munich Technical University
Ismaninger Strasse 22
81675 Munich
Germany

Daniel Scherman
Chemical and Genetic Pharmacology
INSERM U266 – CNRS FRE 2463
Université René Descartes
4, Avenue de l'Observatoire
75270 Paris Cédex 06
France

Martin Schleef
PlasmidFactory GmbH & Co. KG
Meisenstrasse 96
33607 Bielefeld
Germany

Torsten Schmidt
PlasmidFactory GmbH & Co. KG
Meisenstrasse 96
33607 Bielefeld
Germany

Birgit Spänkuch
Department of Gynecology and
Obstetrics
School of Medicine
University of Frankfurt
Theodor-Stern-Kai 7
60590 Frankfurt
Germany

Ulrike Stein
Max-Delbrück-Center for Molecular Medicine
Robert-Rössle-Strasse 10
13092 Berlin
Germany

Klaus Strebhardt
Department of Gynecology and Obstetrics
School of Medicine
University of Frankfurt
Theodor-Stern-Kai 7
60590 Frankfurt
Germany

Oleg Tolmachov
Gene Therapy Research Group
Imperial College
Exhibition Road
London SW7 2AZ
United Kingdom

Capucine Trollet
Chemical and Genetic Pharmacology
INSERM U640 – CNRS UMR 8151
Université René Descartes
4, Avenue de l'Observatoire
75270 Paris Cédex 06
France

Jouni Uitto
Department of Dermatology and Cutaneous Biology
Jefferson Institute of Molecular Medicine
233 South 10th Street, Suite 450
Philadelphia, PA 19107
USA

Carsten Voß
Technology Department
University of Bielefeld
Universitätsstrasse 25
33615 Bielefeld
Germany

Britta Wahren
Department of Virology
Swedish Institute for Infectious Disease Control
Karolinska Institute
17182 Solna
Sweden

Wolfgang Walther
Max-Delbrück-Center for Molecular Medicine
Robert-Rössle-Strasse 10
13092 Berlin
Germany

Abbreviations

$\Delta\Psi_i$	induced cell transmembrane potential
$\Delta\Psi_0$	resting cell transmembrane potential
$\Delta\Psi_t$	threshold cell transmembrane value
AAT	α-1-antitrypsin
AAV	adeno-associated virus
ADA	adenosine deaminase deficiency
AGE	agarose gel electrophoresis
APC	antigen-presenting cell
APIs	active pharmaceutical ingredients
ATA	aurintricarboxylic acid
BÄK	"Bundesärztekammer" (Germany)
BCA	bicinchoninic acid
BMP-4	bone morphogenetic protein 4
CAR	coxsackie and adenovirus receptor
CAT	chloramphenicol acetyl transferase
CBER	Center for Biologics Evaluation and Research (USA)
ccc	covalently closed circular
CCCD	conductively connect charge-coupled device
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
CGE	capillary gel electrophoresis
CIA	collagen induced arthritis
CMV	cyto megalo virus
COPROG	copolymer-protected gene vector
CpG	CpG dinucleotide
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte antigen 4
DC	dendritic cell
DC-Chol	3 beta (N(N′,N-dimethylaminoethane)carbamoyl) cholesterol
DEAE	diethylaminoethyl-

DH	Department of Health (UK)
DMF	drug master file
DMPE	dimyristoyl phosphatidylethanolamine-
DMRIE	1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethylammonium bromide
DOPE	dioleoylphosphatidylethanolamine
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane
DOTMA	N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride
DPI	dry powder inhaler
EAV	equine arteritis virus
ECT	electrochemotherapy
EGF	epidermal growth factor
EGT	electrogenetherapy
EHD	electrohydrodynamic
ELISA	Enzyme-linked immunosorbent assay
EMEA	European Agency for the Evaluation of Medicinal Products
EPI	epidermal powder immunization
EPO	erythro poietin
eqIL-2	equine interleukin 2
ESOPE	European Standard Operating Procedures for Electrochemotherapy and Electrogenetherapy
FDA	Food and Drug Administration (USA)
GAM	gene activated matrix
GCV	ganciclovir
GeMCRIS	Genetic Modification Clinical Research Information System
GFP	green fluorescence protein
GM-CSF	Granulocyte-macrophage colony stimulating factor
GTAC	Gene Therapy Advisory Committee
GTEG (EMEA)	Gene Therapy Expert Group
GLP	good laboratory practice
GMO	genetically modified organism
GMP	good manufacturing practice
GTA	gene transfer agent
GT-MP	gene therapy medicinal product
HA-2	hemagglutinin subunit 2
HCG	Human Genetic Commission
hFIX	human factor IX
HGF	human growth factor hepatocyte growth factor
hnRNP	heterogeneous nuclear ribonucleoprotein

hSeAP	human secreted alkaline phosphatase
HSV-TK/HSVtk	herpes simplex thymidine kinase
HV	high voltage
ICH	International Conference on Harmonisation
i.d.	intradermal
IFN-γ	gamma interferone
IgG1	immunoglobuline G1
i.m.	intramuscular
IND	investigational new drug
IPC	in-process control
KSG	Kommission Somatische Gentherapie (Germany)
LAL	*Limulus amebocyte* lysate
LIF	laser-induced fluorescence
LPD	lipid/polycation/DNA
LPS	lipopolysaccharide
LV	low voltage
MAR	matrix attached region
MART-1	melanoma antigens recognized by T cells 1
MC	muscle cell
MCB	master cells bank
MDI	metered dose inhaler
mEpo	murine erythropoietin
MHC	major histocompatibility complex
MMP-3	matrix metalloproteinase-3 gene
MTC	magnetic targeted carrier
NFκB	anti-apoptosis mediator
NIH	(US) National Institute of Health
NLS	nuclear localization sequence
NLS (Both Ch 5)	nuclear localization signal
NOAEL	no-observed-adverse-effect level
NPC	nuclear pore complex
nt	nucleotide
NT	neutralization test
OBA	Office of Biotechnology Activities
oc	open circular
ORF2	open reading frame 2
ori	origin of replication

PCR	polymerase chain reaction
PDE	permitted daily exposure
pDNA	plasmid DNA
PEG_{5000}	polyethylene $glycol_{5000}$
PEI	polyethylenimine
PLGA	poly(lactide-co-glycolid)
PLK1	polo-like kinase 1
RAC	Recombinant DNA Advisory Committee
SALT	skin-associated lymphoid tissue
SCA1	spinocerebellar ataxia type 1
SCID (mice)	severe combined immune deficiency
shRNA	short hairpin RNA
siRNA	small interfering RNA
SOP	standard operating procedure
SV40	simian virus 40
TCR	T cell receptor
T_E cell	effector T cell
T_H cells	helper T cell
Th-1/2	T helper 1/2
TNF-α	tumor necrosis factor (α)
TLR	Toll-like receptor
TSE	transmisible spongiform encephalopathy
VEGF	vascular endothelial growth factor
WCB	working cells bank

1
DNA Vaccines – An Overview

Britta Wahren and Margaret Liu

1.1
Rationale for DNA Vaccines

Administration of genes via DNA or RNA may be considered the next-generation of scientific development following the use of recombinant proteins for prophylactic vaccines or for therapy. The use of DNA vaccines for the generation of immune responses arose from efforts to find immunogens that would be able to overcome some of the limitations of other modalities of vaccination. With the discovery of the potential widespread applications of DNA plasmids came appreciation of certain of the characteristics of DNA as a product: namely, its advantages, relative to other biologicals, for manufacturing (Chapter 3), product characterization, storage (Chapter 3), and delivery (Chapters 5–12).

From the standpoints both of therapeutics and of vaccines, the use of DNA arose from the desire to have a protein be produced *in situ*. For a variety of applications, ranging from cytokine administration to gene therapy for metabolic and inherited disorders, it was clear that administration of the gene rather than the protein could have multiple advantages: proteins synthesized *in situ* from DNA could potentially persist locally or systemically for longer periods of time without the toxicities associated with the high levels of intravenously administered proteins, certain proteins such as cytokines could be administered to the desired site (i.e., intratumorally) (Chapter 7) more readily when administered as genes, and a protein synthesized from the gene would have mammalian posttranslational modifications, thus avoiding one of the significant challenges that can arise when making recombinant proteins in nonmammalian hosts.

Although vaccines have been considered perhaps the greatest human health achievement, being successful even to the point of eliminating an entire wild-type disease from the planet (smallpox), certain diseases have remained unconquered by vaccination. Two key reasons for this are that the traditional approaches have either simply not worked, or have been considered potentially too risky for a disease such as HIV. As an example, although live attenuated virus vaccines have been extremely effective against a variety of diseases, they have at least the theoretical

risk of reversion to wild type, which in the case of HIV would render the vaccinee infected with a virus that causes what today is still a fatal infection.

As understanding of immune responses to disease increased, it became clear that the use of vaccines that induced primarily antibody responses might not be able successfully to target diseases that required a strong CD8+ T cell responses. Proteins that enter the cellular processing pathway resulting in the generation of CD8+ T cell responses generally have to be endogenously synthesized within a cell. Means to deliver the gene for an antigen, rather than the antigen itself, directly into cells were therefore sought, as the latter would generally result in the exogenous protein being taken into the endolysosomal processing pathway, with the resultant generation of MHC Class II-restricted CD4+ T cells rather than CD8+ T cells. The observation that plasmid DNA could directly transfect cells *in vivo* [1] came as a surprise given the complexity of viral structures that are designed for infecting cells. The process of DNA transfection is very inefficient and, moreover, the best transfected cell type is the muscle cell. Myocytes lack the immune accessory surface molecules needed to activate immune-responding cells appropriately, so it was a surprise to find that direct transfection of myocytes by immunization with unformulated plasmid DNA could indeed result in the generation of CD8+ T cells and protection against a lethal viral challenge [2].

DNA vaccines had further appeal as a product, in additional to their immunologic rationale. The manufacturing process promised to be fairly generic in comparison with those for other biologicals. Traditional live virus vaccines require years of challenging work to attenuate the pathogen properly and to design a cellular production system. Even recombinant proteins can be challenging, because of the need to find the correct producer cell able to make the antigen in the correct form (such as with the correct folding or posttranslational modifications). Because DNA vaccines are bacterial plasmids, the production is quite similar for different vaccines because they differ only in the gene sequence encoding the antigen. The majority of the plasmid, such as the backbone, can be identical or similar. Moreover, DNA vaccines at their simplest, being just plasmids, are potentially more stable (Chapter 3) than live viruses, an attribute that should facilitate their use in resource-poor settings.

1.2 Preclinical Proof of Concept

The initial demonstration that direct immunization with a simple plasmid of DNA encoding a protein from a pathogen could not only result in the generation of both arms of the immune response (cytotoxic T lymphocytes as well as antibodies), but could also protect from an otherwise lethal challenge [2] opened up the field of DNA vaccines. The ability to protect animals from a strain of virus different from the strain from which the gene was cloned generated considerable interest because it offered a potential means to make vaccines for diseases that have multiple strains, such as influenza or HIV. The influenza vaccine, for example, has to contain antigens

for three strains and needs to be reformulated each year as new strains arise. Not only is this a cumbersome process making the adequate yearly supply of vaccines problematic, but such a vaccine does not protect against the epidemic strains differing from the strain in the vaccine that occasionally arise mid-season. Of even more concern is the fact that such a vaccine will not protect against novel pandemic strains of influenza that periodically may arise, most notably in the 1919 Spanish influenza that killed millions of people worldwide. The demonstration that a DNA vaccine made from the genetic sequence of one strain was able to protect against challenge not just with a slightly different drifted strain, but against a different subtype, raised hopes for the ability of DNA vaccines to be effective against a variety of diseases.

From those initial studies, the scientific literature rapidly grew to thousands of publications demonstrating the ability of DNA vaccines to induce immune responses and protective and therapeutic benefits in a variety of preclinical disease models. These models not only included various infectious diseases, including those caused by viruses, bacteria, and parasites, but also encompassed other types of disease, such as cancer, allergy, and autoimmunity (reviewed in [3, 4]). Additional applications for autoimmune diseases and allergies are based upon the ability of the DNA to alter the type of generated T cell help specifically for the particular protein antigen. Autoimmune responses are thought to be due to the inappropriate overproduction of either T helper 1- or T helper 2-type responses. In animal models, DNA vaccines have been shown to be able to alter the form of T cell help, and DNA vaccines have thus been able to prevent or ameliorate the disease in preclinical models of asthma [5] and diabetes [6].

It soon became evident, however, that DNA vaccines, while robust in small animal models, were less immunogenic in nonhuman primates and humans (reviewed in [3, 4]). This has given rise to a variety of approaches for making DNA vaccines of increased potency, as is explored below.

1.3 Clinical Trials

Clinical trials have been performed for DNA vaccines encoding antigens from pathogens and tumors. In addition, however, trials have been performed with DNA encoding therapeutic proteins where not an immune response, but rather expression of the therapeutic protein, is desired. Such studies have included the therapeutic administration of a gene encoding a normal growth factor such as Fibroblastic Growth Factor, or other growth factors, the intent being not to replace a defective or missing protein, but rather to administer a supraphysiologic amount of the growth factor to a local site for a period of time more prolonged than would be achievable by administration of the recombinant protein [7, 8]. The factor then induces the growth of new blood vessels to ameliorate the ischemic condition of the limb or myocardium. DNA has also been used for what is more traditionally considered to be the purview of gene therapy: DNA encoding a form of the muscle

protein dystrophin, for example, has been administered to patients with forms of muscular dystrophy who are lacking in the production of any (or any normal) dystrophin ([9], Chapter 11). In both of these types of clinical applications, the hope is that no immune responses against the therapeutic protein will be generated. In the case in which the DNA is intended to provide additional amounts of a therapeutic protein locally, the individual is already tolerized to the protein, so the administration of the gene through the use of a plasmid should not break the tolerance. The use of a DNA plasmid is thought to be potentially less immunogenic for these purposes than the use of viral vectors, another widely studied approach.

Of course, the most important observation in all the vaccine and therapeutic clinical trials has been that the vaccines have been safe to administer. Secondly, antibody and cellular immune responses, albeit generally low, have been observed in the patients in clinical trials. Interestingly, in HIV patients with long exposure to high levels of viral antigens (due to their high viral loads), new antibody but particularly T helper and cytolytic T cell responses were seen after DNA immunization [10, 11], the DNA somehow eliciting immune responses that the virus could not. This represents the important observation that different methods of producing an antigen *in vivo*, or the effects of different vectors, may result in different immune responses, an observation consistent with the results of preclinical prime-boost studies (see below).

1.4 Second-Generation Vaccines

Perhaps the simplest approach to increasing the potency of DNA vaccines has been to design the plasmids to produce more protein antigen [12] and/or to increase the doses used in clinical trials, even up to milligram doses per vaccine [13, 14]. Another approach, described more fully in this book, is to formulate the DNA in such a way as to facilitate its uptake into cells, or to protect it from degradation. Alternative delivery modalities, such as combining injection (Chapters 6, 7 and 10) with *in vivo* electroporation (Chapters 11 and 12) to increase the amount of transfection, are also being explored.

The coding sequences of DNA vaccines have also been modified to include genes encoding cytokines or other molecules that may enhance immune responses. Because the bacterial DNA in DNA vaccines has sequences that activate Toll-like receptors, the DNA is not simply an inert carrier of the genes, but itself also activates the innate immune system, which may in turn augment the cognate immune responses (reviewed in [15]). Efforts to increase this innate immune stimulation by increasing the number of CpG motifs in the plasmid have met with limited success, but the principal of harnessing the innate immune response to aid in the antigen-specific response is the focus of considerable attention.

DNA vaccines have also been delivered by a variety of routes, variously to increase potency, to generate specific forms of immunity (e.g., mucosal), or to facilitate delivery. The earliest demonstration of the ability of DNA plasmids to generate

antibody responses utilized a 'gene gun' to propel DNA-coated gold beads into the cells of the skin (Chapter 10) [16]. This approach has successfully resulted in the generation of antibodies against hepatitis B surface antigen in clinical studies [17]. In these studies, the titers were lower and required more immunizations that with the licensed protein vaccine, but nevertheless demonstrated the desired immune response in humans. Importantly, though, even patients who had not responded well to the traditional recombinant protein vaccine responded to the DNA vaccine [18]. Additional means of delivery have included the production of biodegradable to which the DNA is adhered (reviewed in [19]) or particles containing the DNA for oral delivery [20] (Chapters 5 and 8). Additional devices that propel the free DNA directly into the skin [21] or mucosa [22] have been developed. *In vivo* electroporation to increase the number of cells that are transfected is also being developed [23] (Chapters 11 and 12).

One of the most promising approaches has been the combination of DNA vaccines with viral vectors or recombinant protein [24, 25] (reviewed in [4]). In this approach a DNA plasmid encoding a given antigen is injected, and the subsequent immunizations then utilize a heterologous delivery system such as a viral vector encoding the same antigen, or a different form of the antigen (e.g., a recombinant protein). This has been referred to as the 'prime-boost' approach. While the mechanism for its efficacy has not been completely determined, a variety of different viral vectors, including adenoviruses and pox vectors, have been utilized. Interestingly, it appears that the approach is most effective when the DNA vaccine is given first, rather than the other way around.

1.5 Conclusions

Although the second generation of DNA vaccines includes more complex formulations and devices, the inherent simplicity of the core of the vaccine (i.e., the plasmid DNA) nevertheless remains an attraction. For scenarios in which the formulation of final product may be more complex (such as the inclusion of two different vectors), it is felt that if that is what is required to overcome the challenges of making a vaccine for HIV, this will nevertheless be a critical part of the medical armamentarium. The potential for developing a somewhat generic, even if complex, approach to a variety of diseases, including diseases that have hitherto been resistant to prevention or therapy, makes these studies of continued high interest.

References

1 Wolff, J. A., Malone, R. W., Williams, P., et al., *Science* **1990**, *247*, 1465–1468.
2 Ulmer, J. B, Donnelly, J. J., Parker, S. E., et al., *Science* **1993**, *259*, 1745–1749.
3 Srivastava, I. K., Liu, M. A., *Ann. Int. Med.* **2003**, *138*, 550–559.
4 Liu, M. A., *J. Intern. Med.* **2003**, *253*, 402–410.
5 Jarman, E. R., Lamb, J. R., *Immunology* **2004**, *112*, 631–642.
6 Prud'homme, G. J., *Expert Rev. Vaccines* **2003**, *2*, 533–540.
7 Baumgartner, I., Isner, J. M., *Ann. Rev. Physiol.* **2001**, *63*, 427–450.
8 Comerota, A. J., Throm, R. C., Miller, K. A., et al., *J. Vasc. Surg.* **2002**, *35*, 930–936.
9 Romero, N. B, Braun, S., Benveniste, O., *Hum. Gene Ther.* **2004**, *15*, 1065–1076.
10 Calarota, S. A., Kjerrstrom, A., Islam, K. B., Wahren, B., *Hum. Gene Ther.* **2001**, *12*, 1623–1637.
11 Calarota, S., Bratt, G., Nordlund, S., *Lancet* **1998**, *351*, 1320–1325.
12 zur Megede, J., Chen, M. C., Doe, B., et al., *J. Virol.* **2000**, *74*, 2628–2635.
13 MacGregor, R. R., Ginsberg, R., Ugen, K. E., et al., *AIDS* **2002**, *16*, 2137–2143.
14 Le, T. P., Coonan, K. M., Hedstrom, R. C., et al., *Vaccine* **2000**, *18*, 1893–1901.
15 Klinman, D. M., Yamshchikov, G., Ishigatsubo, Y. J., *Immunol.* **1997**, *158*, 3635–3639.
16 Tang, D. C., DeVit, M., Johnston, S. A., *Nature* **1992**, *356*, 152–154.
17 Roy, M. J., Wu, M. S., Barr, L. J., et al., *Vaccine* **2000**, *19*, 764–778.
18 Rottinghaus, S. T., Poland, G. A., Jacobson, R. M., et al., *Vaccine* **2003**, *21*, 4604–4608.
19 O'Hagan, D. T., Singh, M., Ulmer, J. B., *Immunol. Rev.* **2004** (June), *199*, 191–200.
20 Howard, K. A., Li, X. W., Somavarapu, S., et al., *Biochim. Biophys. Acta* **2004**, *1674*, 149–157.
21 Trimble, C., Lin, C. T., Hung, C. F., et al., *Vaccine* **2003**, *21*, 4036–4042.
22 Lundholm, P., Leandersson, A. C., Christensson, B., et al., *Virus Res.* **2002**, *82*, 141–145.
23 Otten, G., Schaefer, M., Doe, B., et al., *Vaccine* **2004** (June 23), *22(19)*, 2489–2493.
24 Moorthy, V. S., Imoukhuede, E. B., Keating, S., et al., *J. Infect. Dis.* **2004**, *189*, 2213–2219.
25 Epstein, J. E., Charoenvit, Y., Kester, K. E., et al., *Vaccine* **2004** (April 16), *22(13–14)*, 1592–1603.

2
DNA as a Pharmaceutical – Regulatory Aspects

Carsten Kneuer

2.1
Introduction

It is now more than a decade since that the first genetic treatment, of a four-year-old girl named Ashanthi DeSilva, was initiated on Sept. 14, 1990. Although this initial trial was successful and not associated with major adverse reactions, it prompted the development of regulations for gene therapy clinical trials and the definition of minimal requirements for the quality of prospective Gene Therapy Medicinal Products (GT-MPs). To advise the drug administrations in the design of quality criteria for the latter and the definition of the amount of research and development that should be performed to demonstrate safety and efficacy before a GT-MP can be approved, expert committees were formed. In Europe today this is the Gene Therapy Expert Group (GTEG) of the European Agency for the Evaluation of Medicinal Products (EMEA), while in the US the Food and Drug Administration is assisted by its Center for Biologics Evaluation and Research (CBER).

Neither the EMEA nor the FDA has yet approved any gene therapy product for sale, however, and most developments are still in early clinical or even preclinical stages. According to the Journal of Gene Medicine, 63.2% of all 987 gene therapy clinical trials that had been initiated worldwide by July 1, 2004 were classified as Phase I and only 2.8% as Phase II/III or III [1]. The Genetic Modification Clinical Research Information System (GeMCRIS) of the NIH, with only six phase III studies in its 652 records (September 2004), shows the same pattern [2].

This situation clearly indicates that the current practical need for regulations in gene therapy is not in the approval of new drugs, but in the various aspects of preclinical and clinical trials, including the quality and comparability of the trial material. As this field is largely controlled by institutional research ethics committees and governmental bodies with a wide range of duties, additional expert groups were formed to provide scientific support to these (Table 2.1). Such include the Recombinant DNA Advisory Committee (RAC) reporting to the Office of Biotechnology Activities (OBA) of the US National Institute of Health (NIH), the Gene Therapy Advisory Committee (GTAC), which examines applications for gene therapy clinical

Table 2.1 Agencies and expert groups involved in the regulation of gene therapy clinical trials and products in Europe, the US, the UK, and Germany.

Abbreviation	*Agency or Committee Name*	*Country*
Relevant to clinical trial authorization		
RAC OBA NIH	Recombinant DNA Advisory Committee, reporting to the Office of Biotechnology Activities, of the National Institute of Health	US
GTAC DH	Gene Therapy Advisory Committee, of the Department of Health	UK
KSG BÄK	Kommission Somatische Gentherapie of the Bundesärztekammer	Germany
Relevant to gene therapy product approval		
GTEG EMEA	Gene Therapy Expert Group, of the European Agency for the Evaluation of Medicinal Products	Europe
CBER FDA	Center for Biologics Evaluation and Research, of the Food and Drug Administration	US

trials in the United Kingdom, and the Human Genetic Commission (HCG) that is the official advisory body of the UK Government, the German "Kommission Somatische Gentherapie" (KSG) of the "Bundesärztekammer" (BÄK), and others. These commissions provide recommendations to local or national ethics committees on whether or not to approve a particular gene therapy trial. For example, a RAC review process has to be completed in the US before participants can be enrolled in experiments involving the deliberate transfer of recombinant DNA, or DNA or RNA derived from recombinant DNA, into human research participants. Additionally, current legislation requires registration with and, in some countries, approval by the higher drug authorities.

Finally, general biosafety regulations need to be regarded in the design of production, storage, and trial facilities as well as procedures for transport and disposal of the genetic material and the genetically modified organism to be used.

When planning an individual clinical trial with a new gene therapy product it will therefore be necessary to take the specific guidelines of these national bodies into account. However, with the ultimate goal of marketing approval in mind, it is also essential to consider the stronger criteria of the FDA and EMEA drug administrations for quality of the investigational gene therapy material and both preclinical and clinical study design.

2.2
Quality Requirements for DNA used as a Gene Therapy Product

2.2.1
Introduction

In the European Union, the European Agency for the Evaluation of Medicinal Products (EMEA) has issued a guideline that specifically addresses the quality requirements for gene therapy medicinal products for use in clinical trials, entitled CPMP/BWP/3088/99 "Note for Guidance on the Quality, Preclinical and Clinical Aspects of Gene Transfer Medicinal Products" [3]. This guideline makes a fundamental distinction between plasmid DNA products, nonviral vectors, and viral vectors. In the US, guidance is provided by the FDA document "Guidance for Industry: FDA Guidance for Human Somatic Cell Therapy and Gene Therapy" [4], although DNA preparations used as preventive vaccines are not covered by this document. Separate guidance on these products is available from the Office of Vaccines Research and Review document "Points to Consider on Plasmid DNA Vaccines for Preventive Infectious Disease Indications" [5], though there is some overlap.

In these guidelines it is acknowledged that our clinical experience with such drugs is limited, so "a flexible approach to the control of these products is being adopted so that recommendations can be modified in the light of experience of production and use and of further developments" [3]. Although the recommendations given are generally applicable, all new drug entities will be considered in a case-by-case manner (e.g., particular standards may be expected for DNA vaccines intended for prophylactic use in a large number of healthy individuals).

In addition to CPMP/BWP/3088/99, other notes that may not be specifically targeted for GT-MPs offer guidance for specific aspects of production, quality control, and safety studies for plasmid DNA. These are also discussed in the appropriate paragraphs.

2.2.2
Production and Purification

The materials and procedures used for the production and purification of plasmid DNA are the major determinants of final product quality. For this reason, all raw materials employed in the production and purification of plasmid DNA have to be described and standardized, and their quality must be controlled and documented in accordance with GLP and GMP rules. The same applies to the procedures, so SOPs (Standard Operating Procedures) must be designed and compliance with these should be documented according to GLP and/or GMP rules.

2.2.2.1 Raw Materials

Special attention should be given to the selection of all raw materials, as they may represent potential impurities in the final product. Use of materials associated with a risk of transmitting spongiform encephalopathy (TSE), such as bovine serum

albumin, enzymes, gelatin, or other ingredients for culture media derived from animal tissue, should be avoided. Generally, material from non-TSE-relevant species should be preferred. If there is no choice, the rationale for using this material must be explained and consideration must be given to all measures appropriate to reducing the risk of TSE transmission. The draft document EMEA/410/01 "Note for Guidance on Minimising the Risk of Transmitting Animal Spongiform Encephalopathy Agents via Human and Veterinary Medicinal Products" may be consulted for suitable precautions [6].

2.2.2.2 **Antibiotics**

It is recommended by both the FDA and the EMEA that penicillin and other beta-lactam antibiotics be avoided during production, due to the risk of serious hypersensitivity reactions in patients [3, 4]. If antibiotic selection is used during production (see Chapter 3), it is further preferable not to use selection markers that confer resistance to antibiotics in significant clinical use, in order to avoid unnecessary risk of the spread of antibiotic resistance traits to environmental microbes. The CBER, for example, advises the use of an aminoglycoside antibiotic such as kanamycin or neomycin [5]. These are not extensively used in the treatment of clinical infections, due to their low activity spectra, the prevalence of kanamycin-resistant bacteria, and their problematic therapeutic indexes. Residual antibiotic in the final product should be quantified when possible, and the potential for allergy considered. Consequently, labeling may be required if antibiotics are used during manufacture. As a general rule, nonantibiotic selection systems are clearly preferred.

2.2.2.3 **Solvents**

As with all raw materials employed during manufacture of plasmid DNA, solvents may represent another origin of impurities and should generally be removed to the greatest extent possible as they present no therapeutic benefit. The ICH consensus guideline CPMP/ICH/283/95 entitled "Impurities: Residual Solvents" classifies solvents into three groups. Class 1 solvents are those associated with unacceptable toxicities such as geno- or reproductive toxicity, and include benzene or tetrachloromethane. Their use should be strictly avoided unless justifiable by a risk–benefit analysis, and their concentrations must be kept below the limits given in the guideline. Class 2 solvents include those associated with less severe types of toxicities, such as methanol, chloroform or tetrahydrofuran, while Class 3 solvents are those regarded as less toxic (ethanol, acetic acid). A complete list of classified solvents is included in the above guideline, and permitted daily exposure (PDE) limits for Class 2 solvents are also provided. These values can be used to calculate individual acceptable residue limits. For Class 3 solvents, a collective PDE of 50 mg · day^{-1} can be assumed, and adherence to this limit may be shown by unspecific tests such as loss on drying [7].

2.2.2.4 **Fermentation**

The final quality of any biotechnologically derived product, including isolated plasmid DNA, will be critically influenced not only by the raw materials, but also

by fermentation conditions. It is therefore essential that growth conditions be consistent from batch to batch. Relevant in-process controls should be implemented and the generated data collected as part of the product documentation. It is recommended that a maximum level of cell growth is defined, based on information about the stability of the host/plasmid system, including plasmid copy number, plasmid retention, and yield [3]. Definition of acceptance and rejection criteria will contribute to the stability of yield and quality of the final product, and avoid unnecessary investments in purification and characterization of inferior material.

2.2.2.5 Purification

Methods used to purify the plasmid DNA should be described in detail, justified, and validated; this includes in-process controls and specification limits. Relevant contaminants that should be considered are undesired nucleic acids (RNA, chromosomal host DNA, linear and denatured plasmid DNA; see Chapter 3), host cell proteins, carbohydrates, endotoxins, and impurities introduced during production and purification. Special attention should be given to the removal of endotoxins, also covered in separate FDA and EMEA guidelines.

In many cases, purification is performed by use of an all-in-one third-party solution or even a third party service. Deposition of a drug master file (DMF) with the authorities by the manufacturer, describing the purification system used, can be advantageous for both sides: it allows the user to reference the material simply without prior disclosure of the contents of the file to that customer.

2.2.3 Cell Banking System Procedures

2.2.3.1 Generation and Characterization of Master and Working Cell Banks

Cell banking systems are generally indicated for products that are made repeatedly from the same source, such as bacterial cells producing a plasmid. These cell stocks should be handled by a formal cell banking system, often a two-tiered system consisting of Master and Working Cell Banks (MCBs and WCBs). Specific guidance for the establishment of such MCBs and WCBs is provided in the FDA guideline "Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals" and the adopted ICH guideline CPMP/ICH/294/95 "Quality of Biotechnological Products: Derivation and Characterisation of Cell Substrates Used for Production of Biotechnological/Biological Products" [8, 9]. Essentially, the following points must be considered for master cell banks:

- A description of origin and history of the cells should be provided.
- The procedure for freezing and for recovering the cells should be described. Components used (such as DMSO or glycerol) and the number of vials preserved in a single lot and the storage conditions should be specified.
- The identity of the cells should be confirmed by appropriate genotypic and/or phenotypic markers, and the fraction of the cell population having such identity

markers should be measured as an indication of purity. In the case of transformed cells, vector retention and identity should be confirmed by restriction mapping. CPMP/BWP/3088/99 requires that the sequence of the entire plasmid be established at the stage of the MCB [3].

- MCBs should further be shown to be free of contaminating biological agents, including fungi, viruses, mycoplasma, and bacteria other than an intended bacterial host strain. Testing for bacteriophage is not required, but the possible presence of bacteriophage should be considered, since it could adversely affect stability and yield.
- The product development plans should include data demonstrating for how long and under what conditions the cells can remain frozen and still be acceptably active when thawed (expiration dating). This should be supported by repeated tests of viability, cell identity, and function after thawing and/or expansion. The yield of viable cells and of quantitative functional equivalents should be compared to those values before freezing. "Sterility" should be confirmed by use of aliquots of the frozen cells.

Working Cell Banks, if used, need to undergo only a limited testing program for identity by phenotypic or genotypic markers. Plasmid retention and identity should be confirmed as in MCBs by restriction mapping. They should also be shown to be free of microbial contamination. An extended culture of end-of-production cells may be performed once in the development phase to evaluate whether new contaminants are induced by growth conditions or if vector integrity is compromised.

2.2.4
Product Characterization and Quality Criteria

Various guidelines concerning the required level of product quality testing and recommended quality criteria of final drug products and investigational new drugs have been issued by the US and European Authorities. Those most directly relevant to plasmid DNA-based gene therapy products include the FDA's "Guidance for Industry – FDA Guidance for Human Somatic Cell Therapy and Gene Therapy" and the EMEA guideline CPMP/BWP/3088/99 [3, 4]. Neither document is targeted only for plasmid DNA vectors, and not all of the recommended tests listed will be applicable.

Quantitative assay methods of adequate specificity and sensitivity should be validated by testing of known amounts of reference lots or spiked samples, and data documenting assay performance must be collected. In addition, a distinction will have to be made between the bulk product plasmid DNA (drug substance) and the final formulation, the drug product, if pharmaceutical formulation is intended. Otherwise, only a single set of the tests outlined below is necessary.

2.2.4.1 **Identity**

The isolated bulk material should be routinely tested for identity by methods such as restriction enzyme mapping with multiple enzymes. Alternatively, a specific polymerase chain reaction (PCR) set may be performed on the drug substance. In the case of a facility making multiple constructs, it should be verified that the identity testing is capable of distinguishing between the constructs and detecting cross-contamination. CPMP/BWP/3088/99 further recommends that the entire sequence of the plasmid be determined at least once at this stage, with consideration also of the potential existence of sequence heterogeneity.

2.2.4.2 **Purity**

Obviously, total DNA content of the bulk product will be a major quality criterion. This may be determined by measurement of optical absorbance at 260 and 280 nm. Secondly, homogeneity of size and structure (e.g., supercoiled versus linear forms) should be tested by agarosegel electrophoresis or other suitable chromatographic techniques. If different molecular forms are present, these must be identified and the proportion of supercoiled DNA determined. The level of contamination with RNA or host DNA should be determined. This may be achieved by gel electrophoresis including tests with bacterial host-specific probes. Proteins, if present as a contaminant, may be quantified in silver stained gels. Enzyme-linked immunosorbent assay (ELISA) or Western blotting may be useful to detect contaminating specific marker proteins. As discussed in the section on raw materials, specific tests for known toxic materials involved in production are implicated.

For each contaminant, including undesired molecular forms or modifications, an acceptable degree of contamination should be justified and criteria for acceptance or rejection of a production batch must be established.

2.2.4.3 **Adventitious Agents**

Although contamination with adventitious agents originating from known or unknown sources is primarily a major issue in the production of viral vectors from producer cells, they may also be generated during the fermentation process. Sterility tests should therefore be designed to detect both aerobic and anaerobic bacteria and fungi. Mycoplasma and virus testing is not required for plasmid DNA products, but bacteriophage testing of the master and/or working cell banks may be considered as discussed above.

2.2.4.4 **Potency**

Bacterial modifications to the plasmid DNA structure, such as methylation of promoter regions, and changes in the molecular form exemplified by different degrees of supercoiling may affect the potency of the drug substance, so potency assays should be designed and validated during the product development process. Expression of the inserted gene can be determined by transfection of appropriate cells and demonstration of the active gene product by an appropriate assay, characterized with regard to its sensitivity and specificity. Whenever possible, a potency assay should measure the biological activity of the expressed gene product,

and not merely its presence. If, for example, enzymatic activity is the basis of the proposed therapy, an enzyme activity assay detecting conversion of substrate into product would be preferred over an immunological assay detecting epitopes on the enzyme. If no quantitative potency assay is available, then a qualitative potency test should be performed.

The final formulation of the drug substance (i.e., the drug product) requires additional testing as described in the national or regional pharmacopoeia. Some characterization of the bulk product may be waived if performed on the final product. This includes tests for endotoxins, potency, and general safety studies. As the formulation of plasmid DNA with, for example, cationic liposomes or polymers critically affects its biological activity, it may also be more appropriate from the scientific point of view to perform efficacy and safety studies on the final product rather than on the bulk material. The following test categories must be included in a parenteral dosing form of DNA: sterility, identity and purity, potency, and endotoxin testing by LAL or any other acceptable assay [10].

2.3 Safety Studies for Clinical Trials

2.3.1 General Considerations

As is the case for all new drug products under investigation, a certain amount of preclinical studies testing will be required to justify a clinical trial in human subjects. The purpose of these studies is: (1) to provide evidence that the drug has therapeutic potential, (2) to elaborate the toxicity profile, and finally (3) to allow a safe starting dose to be calculated. In some countries, including the US, the manufacturer must obtain a special permission exemption from, for example, the FDA before starting to study the product in humans. This exemption is usually called an investigational new drug (IND) application. In the IND application the manufacturer explains how it is intended to conduct the study, what possible risks may be involved, and what steps will be taken to protect patients, and provides data in support of the study.

In addition, approval from a committee of scientific and medical advisors and consumers focusing on protecting persons who may participate in the study must be obtained. This committee may be an Institutional Review Board or an Independent Ethics Committee. In some European countries approval by this review board and deposition of a clinical trial study protocol with the authorities may be sufficient, leaving a higher level of responsibility with the investigator and sponsor of a study. Finally, researchers must inform the persons who may be part of the study about the study's potential risks and benefits, and obtain their consent.

The International Conference on Harmonisation (ICH) has formulated a harmonized guideline derived from regional regulations and from other ICH documents to describe internationally accepted principles for the initiation and conductance of clinical trials. This guideline entitled "General Considerations for

Clinical Trials", which has been adopted in the EU as CPMP/ICH/291/95, also provides a good overview over other relevant ICH documents on efficacy and clinical safety [11]. Important principles and practices to ensure the protection of clinical trial subjects are extensively described in "The Guideline on Good Clinical Practice" [12]. With implementation of this guideline, a general legal requirement for registration/approval and close monitoring of clinical trials will very probably also be created in countries where such does not currently exist.

2.3.2 Conduct of Preclinical Safety Studies

2.3.2.1 Regulations

Various drug authorities have gone to great lengths to try to ensure that preclinical toxicology study requirements guarantee a high level of safety in human clinical trials and are as consistent as possible for the various drug product classes. A modified ICH guideline now describes the general rules for "Non-clinical Safety Studies for the Conduct of Human Clinical Trials for Pharmaceuticals" that have been adopted in all three ICH regions: the US, Japan, and Europe [13]. It provides, for example, details of the appropriate timing and duration of general toxicology studies. However, the diversity of product classes, encompassing small molecule drugs, vaccines, blood products, therapeutic proteins, gene therapy products, and monoclonal antibodies, each with its own pharmacodynamic effects, mechanism of action, and safety concerns, makes a "one toxicology program fits all" approach a scientific impossibility. Therefore, the drug toxicology study requirements for determining safety of first administration of plasmid DNA to man may be different from other product classes. Consequently, the International Conference of Harmonisation has produced the more specific guideline ICH S6, which addresses the "Pre-Clinical Safety Evaluation of Biotechnology Derived Pharmaceuticals" [14]. This document has been adopted in the EU as CPMP/ICH/302/95 and provides general guidance with regard to preclinical toxicology testing for various biopharmaceuticals from synthetic, recombinant, and plasma-derived peptides and proteins to oligonucleotide drugs. Although ICH S6 does not specifically cover DNA vaccines and gene therapy products, it touches various aspects that distinguish biologicals from conventional drugs, including the issues of comparability, higher immunogenicity, and lower stability that are relevant to DNA pharmaceuticals. Two guidelines not harmonized so far refer directly to safety studies on plasmid DNA. These are the FDA's "Guidance for Industry: Guidance for Human Somatic Cell Therapy and Gene Therapy" and the draft EMEA guideline CPMP/SWP/112/98 "Safety Studies for Gene Therapy Products" [4, 15]. With these guidelines, the nature and timing of nonclinical studies may be determined and a preclinical development plan for plasmid DNA pharmaceuticals designed. Finally, a draft FDA guidance entitled "Guidance for Industry and Reviewers: Estimating the Safe Starting Dose in Clinical Trials for Therapeutics in Adult Healthy Volunteers" recommends a methodology for determining a safe clinical starting dose in Phase I trials on the basis of results of preclinical studies [16].

2.3.2.2 Design of an Appropriate Toxicology Program

A number of general principles apply to the toxicology studies required to support a Phase I clinical trial, many of which are outlined in the guidance documents described above. However, there are numerous exceptions to each of these that must be considered for gene therapies on a case-by-case basis. The toxicology studies should be completed in compliance with Good Laboratory Practices (GLP) and the study design must be based on the intended clinical trial. However, it is acknowledged by the authorities that full GLP compliance may not always be possible in the highly specialized test systems for biopharmaceuticals [14]. Such areas of noncompliance must be identified and their relevance assessed. If intended to back up early clinical trials, the studies usually include acute (single-dose) studies and repeat-dose studies, along with an evaluation of genetic toxicology and reproductive toxicology studies that may be part of the repeat-dose study.

2.3.2.3 Single- and Repeat-Dose Toxicity Studies

As a general rule, the acute toxicity of a pharmaceutical should be evaluated in two mammalian species prior to the first human exposure [13], but a dose escalation design is also acceptable instead of single-dose applications. Repeat-dose studies in two species, one of which can be a rodent, while the other one must be a nonrodent, over a minimum of two weeks are generally required, but their recommended duration is usually related to the intended duration of clinical exposure.

Special consideration must be given to the identification of relevant species for evaluation of plasmid DNA (see also below), as results obtained in nonrelevant species will be misleading. When only one relevant species can be identified, or the biology of the investigated DNA is well understood, toxicity studies in only one species may suffice [14]. Tested dose levels should reflect expected species differences in the potency of the DNA and include the maximum proposed human dose as well as additional doses with the aim of determining a no-observed-adverse-effect level (NOAEL) in the repeat-dose study. The route of administration should mimic the clinic, but it is recommended that parenteral administration is also performed to register the toxicity profiles of drugs with low bioavailability and/or low toxic potentials, such as plasmid DNA. The dosing regimen and study duration vary with product class and are outlined in the appropriate guidance documents mentioned above. The toxicology parameters to be evaluated generally include mortality, clinical signs, body weight, food consumption, clinical chemistry, hematology, gross pathology, and histopathology. A part of the treatment groups is usually employed to assess reversibility after 7 or 14 days, but for gene therapy products, the duration of the recovery phase should be based on the persistence of both the DNA and the expression product [15].

Requirements for acute and repeated dose toxicity studies may also be altered if a product development program has “fast-track” designation.

2.3.2.4 Safety of the Formulated Plasmid DNA

Careful consideration should be given to the material to which humans will actually be exposed. If, for instance, the plasmid DNA is complexed with a cationic lipid

preparation, the stability of the drug, the site of transfection, and the degree and duration of transgene expression is likely to be different. The safety of the excipient used to formulate the drug is usually investigated as part of the final drug, unless there are specific concerns about aspects of the material that require additional testing in the absence of nucleic acids.

2.3.2.5 **Specific Safety Considerations**

Concerns relating to all gene therapy products include distribution to tissues other than the desired target tissue and expression of the intended protein there, as well as the concern that DNA sequences might become integrated into the genome (genotoxicity). The results of distribution studies of plasmid DNA vaccines and gene therapy products should therefore also be evaluated against this background. The draft guideline CPMP/SWP/112/98 recommends the inclusion of suitable assays such as quantitative or *in situ* PCR in the distribution studies [15]. It further states that the possibility of distribution to and integration of therapeutic DNA sequences in the genomes of germline cells must be investigated (reproductive toxicity). The issue of germline transduction had seldom been tested in animal models until recently, and although it has not so far been observed in clinical trials, these new studies have renewed concern. In this context, any sequences that may facilitate homologous recombination of plasmid DNA drugs must be justified. A compilation of relevant studies and discussion is provided by meeting reports from the FDA Biological Response Modifiers Advisory Committee and the CPMP Gene Therapy Expert Group [17, 18].

Furthermore, each product class comes with a specific set of safety concerns that must be considered in planning the initial toxicology studies. For example, induction of a specific immune response is inherent in the mechanism of action of any vaccine. Concerns regarding prophylactic DNA vaccines must therefore include induction of "nonspecific" antibodies, local injection site reactions, induction of undesirable cytokine production, IgE induction, inflammatory response, and autoimmunity, among others (immunotoxicity). Interestingly, these specific concerns regarding potential immunotoxicity have been extensively considered in the guideline of the Committee for Medicinal Products for Veterinary Use on DNA vaccines for use in animals [19]. Here it is further acknowledged that, although DNA is of very low immunogenic potential, bacterial DNA sequences can have strong mitogenic and immunostimulatory effects. This property may be used to advantage in DNA vaccines, but incorporation of immunostimulatory sequences should be undertaken with care and reevaluation of product safety.

2.3.2.6 **Choice of Animal Model**

Special consideration needs to be given to the choice of a relevant animal toxicology model, since species used for conventional toxicity tests, such as rat and mouse, may not be appropriate, especially for DNA vaccines. The relevant model should provide the most accurate possible prediction of potential toxicity to humans. For a drug, including plasmid DNA for gene therapy, such a model is one in which this drug is distributed and metabolized in a similar manner as in humans. Furthermore,

if the plasmid DNA is intended as a vaccine, the relevant model must be one in which the encoded antigen is immunogenic. This may be warranted if the appropriate antigenic epitope is expressed in a similar manner as it is in humans. Unfortunately, the existing and drafted FDA and EMEA guidelines provide no specific recommendations on this important issue.

2.4 Special Issues

2.4.1 Comparability of Plasmid Gene Therapy Products

Scale-up of culture and purification processes will occur as the product development progresses from preclinical experiments to late clinical trials and commercial production. Changes in process parameters may have consequences on the overall product quality, affecting both biochemical and biological properties such as purity and potency. Additional testing may be required to determine the comparability of the material employed at the various stages of development. If comparability is limited, further action may be necessary.

Although not specifically written for DNA pharmaceuticals, the best guidance on this issue is provided by the FDA document on "Demonstration of Comparability of Human Biological Products, Including Therapeutic Biotechnology-derived Products" and the corresponding CPMP "Note for Guidance on Comparability of Medicinal Products containing Biotechnology-derived Proteins as Drug Substance" [20, 21]. Both documents identify the comparability of potency and immunogenicity that may be affected by changes in the manufacturing or formulation process as major issue.

To assess the amount of reevaluation that is required, changes in the process should be classified as to whether these have had:

- no impact on quality criteria,
- impact on in-process controls without impact on product specifications,
- impact on quality criteria and no anticipated consequences on safety and efficacy, or
- impact on quality criteria and anticipated consequences on safety and efficacy.

2.4.2 Mixed Plasmid Preparations

For certain gene therapy applications, namely DNA vaccination, the use of preparations consisting of more than one individual type of plasmid may be indicated. In this case, the EMEA requires that all relevant information and safety data be provided for each component of the mixture [3]. Only if scientifically justified may the mixture be characterized as a whole. In this case, however, it should be

born in mind, that any changes in the composition of the mixture may consequently require a costly and time-consuming reevaluation of the product.

2.4.3 Plasmid Molecular Structure

From previous experience with drug stereoisomers, all regulatory bodies will require that, if the intended therapeutic effect is based on a particular molecular species, this species should either be isolated or enough structural and biological information provided to show that the appropriate and biologically active form is present and at what content.

Plasmid-derived DNA species such as linear and relaxed circular DNA may be less effective in expressing the inserted antigen gene, so a specification for the minimum amount of supercoiled DNA should be present. This parameter will also be a major criterion measured during stability studies.

2.5 Biosafety Issues and Environmental Risk Assessment

Human gene therapy necessarily involves the use of recombinant genetic material such as plasmid DNA for transfer of genetic information and genetically modified organisms (GMOs) for large-scale production of this material. National biosafety regulations need to be considered according to the risk group into which the involved GMOs were classified. For production of plasmid DNA this will usually be risk group 1. This necessitates authorization of the production facility, appropriate containment of the GMOs, and documentation of all experiments including generation, storage, and inactivation of GMOs. If certain limits are exceeded, such as a culture volume of 10 L in the US, an authorization of the experiment may be necessary. Depending on national legislation, reporting (notification/registration) of the experiment to the competent authority at the time of initiation may also be required in addition to documentation.

Strictly speaking, human gene therapy will itself produce genetically modified organisms – the patients. This case, however, is not covered by general biosafety regulations as it is recognized that: (1) an acceptable gene therapy will be designed as safe for patient and environment, and (2) typical biosafety measures such as lifelong physical containment are not acceptable. In addition, gene therapy experiments lend themselves to another containment mechanism, namely, the application of highly specific biological barriers. These limit the horizontal transmission of a plasmid DNA vector and its dissemination and survival in the environment. The use of appropriately designed plasmids should therefore decrease the probability of dissemination of recombinant DNA outside the human host by many orders of magnitude.

On the other hand, gene therapy with genetically modified viruses will be viewed by some states as deliberate release of GMOs into the environment. This will be

important in terms of the complexity of the administrative procedures required and precautions to be taken to reduce the risk of release of the GMO, such as patient hospitalization. In the US, clinical trials involving human gene transfer must not be started before an NIH-approved Institutional Biosafety Committee has inspected all individual trial sites and given an approval. Fortunately, gene therapy with plasmid DNA is currently not regarded as deliberate release of GMOs, although genetically modified patients may be engineered as discussed above.

For marketing authorization of a final gene therapy medicinal product, an environmental risk assessment may be part of the dossier submitted to the drug authorities.

References

1 Website of The Journal of Gene Medicine, www.wiley.co.uk/genmed/clinical.
2 The Genetic Modification Clinical Research Information System (GeMCRIS) of the NIH Office of Biotechnology Activities, www4.od.nih.gov/oba/RAC/GeMCRIS/GeMCRIS.htm.
3 CPMP/BWP/3088/99 Note for Guidance: Quality, Preclinical and Clinical Aspects of Gene Transfer Medicinal Products, April 24, **2001**.
4 FDA Guideline: Guidance for Industry – FDA Guidance for Human Somatic Cell Therapy and Gene Therapy, March 30, **1998**.
5 Office of Vaccines Research and Review: Points to Consider on Plasmid DNA Vaccines for Preventive Infectious Disease Indications, CBER (301) 594–2090, **1996**.
6 Revision 3 of draft EMEA/410/01 Guideline: Note for Guidance on Minimising the Risk of Transmitting Animal Spongiform Encephalopathy Agents via Human and Veterinary Medicinal Products, June 23, **2004**.
7 ICH Harmonised Tripartite Guideline (Q3C), adopted as CPMP/ICH/283/95: Impurities: Residual Solvents, July 17, **1997**.
8 FDA Guideline: Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals, July 12, **1993**.
9 ICH Harmonised Tripartite Guideline, adopted as CPMP/ICH/294/95: Quality of Biotechnological Products: Derivation and Characterisation of Cell Substrates Used for Production of Biotechnological/Biological Products, July 16, **1997**.
10 FDA Guideline: Guideline on validation of the limulus amebocyte lysate test as an end-product endotoxin test for human and animal parenteral drugs, biological products, and medical devices, December 2, **1987**.
11 ICH Harmonised Tripartite Guideline (E8), adopted as CPMP/ICH/291/95: General Considerations for Clinical Trials, July 17, **1997**.
12 ICH Harmonised Tripartite Guideline (E6), adopted as CPMP/ICH/135/95: The Guideline on Good Clinical Practice, July 17, **1996**.
13 ICH Harmonised Tripartite Guideline (M3) adopted as CPMP/ICH/286/95: Non-clinical Safety Studies for the Conduct of Human Clinical Trials for Pharmaceuticals, November 16, **2000**.

14 ICH Harmonised Tripartite Guideline (S6), adopted as CPMP/ICH/302/95: Pre-Clinical Safety Evaluation of Biotechnology Derived Pharmaceuticals, July 16, **1997**.

15 Draft guideline CPMP/SWP/112/98: Safety studies for Gene Therapy Products, January 28, **1998**.

16 Draft FDA Guideline: Guidance for Industry and Reviewers – Estimating the Safe Starting Dose in Clinical Trials for Therapeutics in Adult Healthy Volunteers, January 16, **2003**.

17 FDA Biological Response Modifiers Advisory Committee (BRMAC) meeting report, May 10, **2002**.

18 EMEA/CPMP/1978/04 Document: CPMP Gene Therapy Expert Group (GTEG) meeting report, February 26–27, **2004**.

19 CVMP/IWP/07/98 Note for Guidance: DNA Vaccines Non-amplifiable in Eukaryotic Cells for Veterinary Use, March, **2000**.

20 FDA: Guidance Concerning Demonstration of Comparability of Human Biological Products, Including Therapeutic Biotechnology-derived Products, Federal Register, April 26, **1996** (61 FR 10426).

21 CPMP/3097/02 Note for Guidance: Comparability of Medicinal Products containing Biotechnology-derived Proteins as Drug Substance – Non Clinical and Clinical Issues, December 17, **2003**.

3
From Bulk to Delivery: Plasmid Manufacturing and Storage

Carsten Voß, Torsten Schmidt, and Martin Schleef

3.1
Introduction

The use of plasmid DNA as a novel class of APIs (active pharmaceutical ingredients) in clinical gene therapy and DNA vaccination trials has increased the demand for innovative techniques and process steps for DNA production in multigram quantities.

3.1.1
Gene Therapy

The principle of gene therapy was first introduced in the 1970s and refers to the transfer of therapeutic genetic material into mammals in order to cure hereditary or acquired diseases. In the early 1990s several efforts to cure certain monogenetic diseases such as adenosine deaminase deficiency (ADA), cystic fibrosis, or gaucher disease were implemented. In the following decade, treatment of acquired diseases such as cancer and cardiovascular diseases became preferred targets for several gene therapy efforts (Mountain 2000). In general, the strategies applied in these trials include the correction of a nonexistent or insufficient gene function or knockout of a detrimental gene expression. For gene delivery, several viral and nonviral delivery systems have been developed. Advantages of viral delivery systems are found in efficient cell targeting, while the main disadvantages are considered to be safety concerns with respect to oncogene activation (Check 2002) and immunogenic shock (Raper et al. 2002). In comparison, no safety concerns arise with nonviral delivery systems such as plasmid DNA and a simple manufacturing process (in comparison with viral vectors) makes them an interesting gene delivery system. However, the efficacy of gene expression is still an issue to be improved.

Currently there are over 900 approved clinical trials worldwide, indications being cancer (66%), monogenic (9%), vascular (8%), and infectious diseases (7%) (www.wiley.co.uk/genmed/clinical/), most of them still being in phase I and only

a few in the final phase III. However, no DNA-based pharmaceutical has yet made the step from bench to market. In most cases viral vectors are applied, with the use of naked plasmid DNA or DNA in combination with other nonviral delivery systems constituting about 25%.

3.1.2 DNA Vaccination

The immunization of animals or humans with genetic material coding antigen is another medical application of nucleic acids. Direct injection of plasmid DNA into mouse muscle resulted in extended *in vivo* expression of the encoded protein (Wolff et al. 1990). The expressed protein was detectable even 60 days after injection, indicating prolonged expression *in vivo* and thus suggesting potential therapeutic applications. In the following years, HIV (Barouch et al. 2000a, 2000b, Mascola and Nabel 2001, Shiver et al. 2002), malaria (Doolan and Hoffman 2001), and hepatitis B and C (Michel et al. 1995, Major et al. 1995) became the preferred targets for DNA vaccine development (overview: Schleef 2001). Plasmid DNA is considered to be superior to conventional protein-based vaccines in terms of production and storage as well as application and safety. These novel DNA-based vaccines contain no protein at all: only the cells transfected with the nucleic acid express the coded antigen, thus resulting in an immune response comparable to a real infection.

3.2 Manufacturing of Plasmid DNA

The use of plasmid DNA in clinical trials and as approved pharmaceutical drugs in the future has caused the development of robust and scalable production processes for DNA manufacturing according to GMP (good manufacturing practice). These processes have to fulfil the requirements of respective guidelines and laws. In general, such processes comprise cultivation of the plasmid-harboring *Escherichia coli* host and subsequent isolation and purification of the product.

3.2.1 Bacterial Cultivation

Besides the use of qualified and well documented production strains for the microbiological amplification of the required plasmid DNA, the cultivation of biomass in fully defined media has become a safety issue with respect to recent discussion on the use of animal-derived raw materials (Schleef and Schmidt 2004).

Process elements and cultivation media are potential sources of contamination. In the past, culture media for the growth of microorganisms were based on undefined beef extracts. One major improvement for such media was the addition

of peptones and salts, which resulted in increased supplementation with amino acids and enhanced osmolarity. These peptones were generated by enzymatic digest of meat (Bridson 1994).

Today's technology for the generation of complex bacterial growth media uses soy bean peptones to avoid animal-derived protein sources in the face of problems caused by BSE or TSE. Generally, in order to avoid BSE risk materials as recommended by regulatory guidelines (EMEA 2001), the use of synthetic growth media should be favored.

To ensure high productivity in cultivation, a large biomass concentration with high plasmid content has to be produced. Generally, these high biomass concentrations are achieved by fed-batch techniques. Such high cell density cultures have been described for a variety of products derived from *E. coli*, including recombinant proteins (Schroeckh et al. 1992), antibodies (Horn et al. 1996), or polyhydroxybutyric acid (Wang and Lee 1998). The feed of concentrated medium may be controlled by monitoring different operating variables in the bioreactor, including pH (Lee and Chang 1994) or dissolved oxygen (Nakano et al. 1997, Schmidt et al. 1999b), or by indirect determination of the specific growth rate (Macaloney et al. 1997] or online monitoring of a limiting substrate (Paalme et al. 1990).

Several processes for plasmid DNA production have been described, most of them aiming only at high biomass and product concentrations. The homogeneity of the plasmid at the cultivation stage is rarely addressed. Reinikainen et al. (1989) examined the influence of pH and temperature on plasmid copy number in cultivations on a semi-defined medium, but no statement was made regarding plasmid homogeneity. Lahijani et al. (1996) described the cultivation of a pBR322-derived plasmid. The copy number of the plasmid was increased by introducing a temperature-sensitive point mutation. Setting the cultivation temperature to 42 °C in the growth phase resulted in a plasmid concentration of 37 mg · L^{-1} in batch experiments on semi-defined medium and 220 mg · L^{-1} in fed-batch experiments. However, the isolated DNA was a nonhomogenous product comprising several multimeric plasmid forms and chromosomal DNA. Additionally, segregative plasmid stability was maintained by supplementation of antibiotics. Schmidt et al. (1999b) described dissolved oxygen-controlled fed-batch cultivation on a defined glycerol medium. A product concentration of 100 mg · L^{-1} and a dry biomass concentration of 48 g · L^{-1} were achieved, resulting in a selectivity of 2.1 mg · g^{-1}.

The cultivation of *E. coli* to high cell densities for plasmid DNA production in a batch mode was described by Voss et al. (2004). With use of a fully defined synthetic glycerol medium, 45 mg · L^{-1} plasmid DNA could be produced, while the selectivity of 2.7 mg · g^{-1} was comparable to cultivations on semidefined media (Figure 3.1). A high plasmid homogeneity was maintained during the whole cultivation process, with more than 90% in the preferred supercoiled form.

For subsequent purification the produced biomass is separated from the culture medium by centrifugation or microfiltration and is stored at low temperatures (–20 °C).

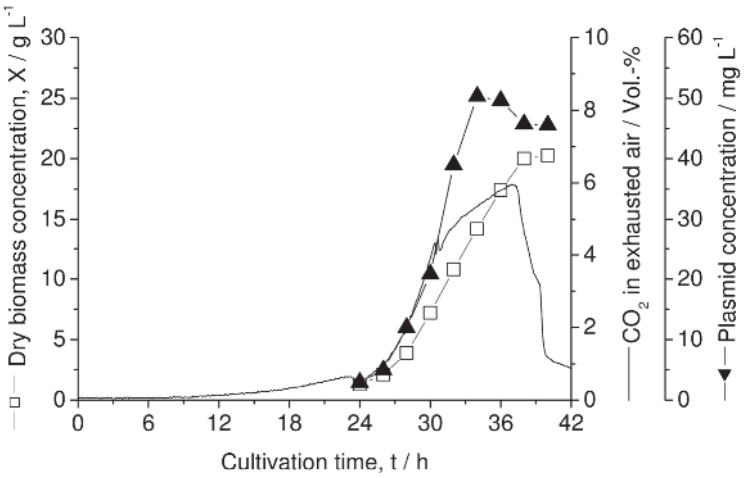

Figure 3.1 Batch cultivation on synthetic glycerol medium supplemented with 37 mmol · L^{-1} ammonium chloride.

3.2.2 Plasmid DNA Purification

High quality requirements for plasmid DNA-based pharmaceuticals, as well as guidelines set by regulatory authorities (EMEA 1998, FDA 1998), resulted in the development of different purification strategies. In most cases plasmid DNA is released from the cells by alkaline lysis (Birnboim and Doly 1979), followed by clarification by centrifugation or filtration, but other procedures such as thermal lysis have also been described (Lee and Sagar 1999, Schumacher et al. 2002). During alkaline lysis, both chromosomal DNA and plasmid DNA are denatured by alkaline pH-shift. A subsequent neutralization step allows reannealing of plasmid DNA within a short period. The chromosomal DNA does not reanneal completely to the native DNA double strand, however, so the major part of the chromosomal DNA is a component of the flaky material generated after neutralization and mainly consisting, together with the DNA, of potassium dodecyl sulfate, insoluble proteins, cell debris, and lipopolysaccharides (LPSs). Chromosomal DNA is extremely shear-sensitive, which may easily result in DNA fragmentation, so the scaling up of cell disruption is one of the crucial steps in the whole purification process. Simple scaling up of alkaline lysis from lab-scale to an industrial stirred tank reactor in batch mode will result in shear forces on plasmid and host cell chromosomal DNA (Levy et al. 2000), thus reducing product concentration and contaminating the product stream. Gentle lysis can be achieved by continuous mixing of biomass suspensions and lysis buffer, followed by neutralization of the lysate in a static mixer (Wan et al. 1998), though this method does not solve the problem of debris removal. For that purpose, time-consuming procedures such as centrifugation or filtration have to be applied, and shear forces during these clarification steps can also result in contamination of the product stream with small fragments of chromosomal DNA.

Table 3.1 Constituents of *Escherichia coli* lysates (Stadler et al. 2004).

Content of bacterial cell lysates	
Proteins	55%
RNA	21%
Host chromosomal DNA	3%
Lipopolysaccharides	3%
Plasmid DNA	3%
Others	15%

Cleared lysates contain only about 3% plasmid DNA, together with impurities originating from the host cells (Table 3.1). The separation of plasmid DNA from host cell impurities comparable to the product in their physical and chemical characteristics is a major challenge for bioprocess engineering, since subsequent purification by chromatographic steps suffers from the low capacities of common stationary phases for nucleic acids (Ljunglöf et al. 1999).

Critical process elements in DNA purification are ribonucleases such as RNase A, which is typically prepared from bovine pancreas. RNase A is able to hydrolyze phosphodiester bonds within RNA molecules. Bacterial RNA is a major contaminant in plasmid production, despite its short lifetime, because it blocks binding capacity in the chromatography steps, and so an enzymatic digestion of RNA prior to chromatographic processing is usually applied (Bussey et al. 1998, Schorr et al. 1999). In pharmaceutical manufacturing processes, the use of bovine RNase is critical. In general, avoiding the use of RNase increases product safety (Schleef and Schmidt 2004).

Different precipitation steps for RNA (Eon-Duval et al. 2003a) and plasmid DNA (Costioli et al. 2003, Horn et al. 1998, Lander et al. 2002, Murphy et al. 1999) have been described. However, these methods are either only applicable at laboratory scales (Lander et al. 2002, Murphy et al. 1999, Costioli et al. 2003) or suffer from high loss of product due to unsatisfactory selectivity (Eon-Duval et al. 2003a, Horn et al. 1998). The extraction of plasmid DNA in aqueous two-phase systems has been described by Ribeiro and coworkers (2002). For efficient partitioning of the DNA into the polyethylene glycol (PEG) phase, high concentrations of PEG and potassium phosphate are necessary, thus making the extraction system extremely susceptible to precipitation at the interface. The application of ultrafiltration only results in the depletion of low molecular weight RNA (Eon-Duval et al. 2003b), while high molecular weight RNA still remains in the retentate together with plasmid DNA. Very selective separation of plasmid DNA from RNA can be achieved by gel filtration in the presence of ammonium sulfate (Lemmens et al. 2003), but this separation technique is limited by the low capacity of gel filtration media and the time-consuming operating conditions. Further purification is usually accomplished by anion-exchange chromatography. Contaminants such as lipopolysaccharides can be further depleted during this step (Colpan et al. 1995,

Horn et al. 1998), but the main problem associated with common anion-exchange matrices is their poor capacity for nucleic acids, due to their porous structures. Different strategies to circumvent this problem have been explored, resulting in the development of monolithic stationary phases for biochromatography (Strancar et al. 2002) as well as the application of small (30 µm) monodispersed microbeads (Stadler et al. 2004) in plasmid purification. Recent research in plasmid purification has also resulted in the development of matrices for selective purification of ccc forms through the use of mercaptopyridyl ligands (Lemmens et al. 2003).

3.2.3 Innovative Aspects in Plasmid Manufacturing

The major bottlenecks in current plasmid purification techniques – gentle cell disruption and RNA removal – have already been outlined above. Different strategies to solve these problems have been investigated recently (Voß 2004).

Continuous alkaline lysis represents a suitable method for the gentle disruption of large amounts of *E. coli* cells. Mixing in a simple T-connector results in efficient cell disruption, so pressure drops associated with mixing in static mixers can be avoided. Separation of cell debris from the liquid after neutralization can be achieved by simple froth flotation (Figure 3.2). Solids content was measured by optical density at 600 nm (OD_{600}), and indicated that no further clarification was necessary prior to subsequent purification.

Extraction is an alternative to chromatographic methods for the removal of RNA because of its scalability and inexpensiveness. Aqueous two-phase systems, however, are susceptible to precipitation of nucleic acids at the interface because of the high concentrations of polymer and salt in suitable systems. Reverse micellar phases have been applied in protein purification (Hatton 1989) and have also already been shown to be well suited for nucleic acid partitioning (Goto et al. 1999). Since salt concentrations in these systems are considerably lower than in aqueous two-phase systems, precipitation at the interface is less likely.

In general, partitioning between the reverse micellar phase and an aqueous phase is governed mainly by pH and ionic strength. We have recently investigated the potential of this extraction procedure for the separation of plasmid DNA and RNA. The results show that distribution can be controlled through the ionic strength of the aqueous phase and that plasmid DNA can be separated from RNA. The reverse micellar phase has a high capacity for nucleic acids, up to 2 mg · mL^{-1} (Table 3.2), superior even to common chromatographic media. Back-extraction with sodium chloride concentration below 0.5 M allows direct application to subsequent purification processes such as anion exchange chromatography.

A different strategy for selective purification makes use of affinity procedures. For DNA purification, triple helix formation is a well known method exploited both in precipitation (Costioli et al. 2003) and in chromatographic separation (Schluep and Cooney 1998). The kinetics of triple helix formation are very slow, however, and the affinity ligands have very poor chemical stability. Recent pub-

Figure 3.2 Bacterial alkaline lysate during (above) and after (below) flotation.

Table 3.2 Capacity of reverse micellar phases made up of isooctane and TOMAC (Voß 2004).

c_{DNA} *before extraction* $[mg \cdot L^{-1}]$	c_{DNA} *in RM phase* $[mg \cdot L^{-1}]$	c_{DNA} *in aqueous phase* $[mg \cdot L^{-1}]$	*Recovery* [%]
50	50.4	0.3	101
100	129.6	0.2	130
150	193.6	0.5	129
200	263.0	0.4	132
400	426.8	0.2	107
600	614.4	0.1	102
800	878.5	0.3	110
1000	991.3	0.4	99
1200	1233.7	0.1	103
1400	1296.1	0.2	93
1600	1534.7	0.2	96
1800	1786.9	0.1	99
2000	2068.9	0.1	103

lications have shown the potential of protein–DNA interaction for selective purification of plasmid DNA (Ghose et al. 2004, Woodgate et al. 2002). Purification methods based on this affinity principle still have to be tested with regard to the chemical and biochemical stability of the ligands, their selectivity for double-stranded nucleic acids, and the binding capacity of stationary phases coupled with such ligands.

3.3 Quality Control of Plasmid DNA Vectors

Plasmid DNA quality mainly depends on the type of manufacturing, storage, and application. The safety of these drugs is dependent on vector construction, characterization, testing by toxicology and functional studies before clinical trials. Driven by the production process, those parameters are well defined, but subjected to ongoing improvements regarding the state of the art in analytical techniques. Table 3.3 shows a selection of relevant quality control tests for in-process control (IPC) and product release. No guideline exists, indicating a certain value or specification for clinical material (except for "sterile" for sterility testing and "identical" in case of DNA sequencing). Regulatory bodies usually require a safe and carefully monitored product, manufactured in a state-of-the-art process.

Table 3.3 Important criteria for quality assurance and quality control of plasmid DNA medicines (selection).

Test	*Analytical method*
DNA concentration	UV absorption (260 nm)
General purity	UV scan (220–320 nm)
Homogeneity (ccc content)	CGE
Purity (visible)	Visual inspection
Purity (chromosomal DNA)	Agarose gel (visual), Southern blot, quantitative PCR
Purity (RNA)	Agarose gel (visual), fluorescence assay, quantitative PCR
Purity (protein)	BCA test
Purity (LPS)	LAL test
Purity (microorganisms)	Bioburden test, sterility test
Identity (vector structure)	Restriction fragment lengths conforms to reference in AGE (1–3 enzymes)
Identity (sequence)	Sequencing (double strand)

3.3.1
Proteins, Ribonucleic Acid, and Lipopolysaccharides

Proteins, RNA, and lipopolysaccharides (LPSs, endotoxins) all constitute major host cell impurities that have to be removed to a minimum concentration during the plasmid DNA purification process. The presence of proteins can be detected by colorimetric assays, such as the Bradford or BCA (bicinchoninic acid) tests.

Quantification of residual RNA is important, since plasmid DNA is purified without the use of RNase. It can be performed directly by fluorescence assays (Ribogreen) after digestion of the plasmid DNA with DNase or after agarose gel electrophoresis. An alternative approach is the determination of RNA by quantitative RT-PCR.

Bacterial LPS endotoxins have pyrogenic effects on mammalian cells, so dramatic reductions in these impurities are necessary for use of the manufactured DNA in research and clinical trials. LPSs can be determined by kinetic measurement of *Limulus amebocyte* lysate (LAL) reaction with endotoxins.

3.3.2
Chromosomal DNA

Host chromosomal DNA is already separated from plasmid DNA during alkaline lysis. However, shear forces during cell disruption and clarification can result in DNA fragmentation. Since these fragments have wide size distributions, detection and separation become difficult. While contamination of plasmid DNA with

chromosomal DNA was previously typically in the 5–10% range, novel purification technologies allow reduction to below 1%. Some chromosomal DNA fragments are large enough to migrate in one distinct band in agarose gel electrophoresis (AGE). Smaller fragments can be detected as undefined smears by overloading the agarose gel. More sensitive assays such as Southern blot hybridization (Southern 1975) can demonstrate this – depending on the hybridization and washing conditions applied. The most sensitive assay is a kinetic PCR method that uses a TaqMan probe to quantify chromosomal DNA contamination (Smith III et al. 1999).

3.3.3 Plasmid Identity

Plasmid DNA should be tested for identity. A simple analytical method for determining plasmid identity is restriction digestion of the plasmid DNA, followed by agarose gel electrophoresis. The length of the restriction fragments can be estimated by comparison with a linear DNA size marker, such as a 1 kb ladder. The determined fragments have to conform to the calculated fragments or to a reference DNA with respect to identity. In our experience, four different enzymes, each with minimum of two restriction sites, should be used.

The integrity of the nucleotide sequence has to be determined by sequencing of the plasmid DNA. Sequencing of the complete plasmid or of only parts thereof has to be evaluated for each individual case.

3.3.4 Plasmid Topology (Structural Homogeneity)

Plasmids of identical nucleotide sequence isolated from *E. coli* may exist in different shapes and forms. The structural homogeneity of plasmid DNA is usually determined by agarose gel electrophoresis (AGE), and different bands in AGE of a plasmid sample may be assignable to different plasmid forms. The assignment of bands to the different topologies is not easy, however, since the electrophoretic mobilities of plasmids of different shape change with the electrophoretic operating conditions (Garner and Chrambach 1992, Johnson and Grossmann 1977, Serwer and Allen 1984, Sinden 1994). In addition, the quantification of forms on the basis of the signal intensities of stained bands in AGE may not be reliable because of nonlinear responses; adequate equipment is required in order to obtain reproducible results.

It is well known that typically only one band, the ccc form, is observed when only a small amount of a plasmid sample is applied to an agarose gel. Standard AGE usually reveals two prominent bands: the ccc form and another, more slowly migrating form, commonly thought to be the oc form. It has been demonstrated (Schmidt et al. 1999a), though, that this is not always the case, since the oc form may comigrate with ccc dimers.

Capillary gel electrophoresis (CGE) allows identification and quantification of all the prominent plasmid topologies discussed (Schmidt et al. 1996, 1999a, overview:

Schmidt et al. 2001). CGE is performed by use of thin (100 µm) coated capillaries 40–60 cm in length filled with a liquid polymer, such as a solution of hydroxypropylmethylcellulose. Electrophoretic separation takes place through the application of a high voltage (5–30 kV) at both ends of the capillary. Special intercalating dyes, such as YOYO, YO-PRO, TOTO, or PicoGreen, enable online detection of the different plasmid forms with high resolution by laser-induced fluorescence (LIF). The automated system offers high reproducibility, reliable quantification, and short analysis times. In contrast with AGE, quantification of plasmid forms by CGE is possible over a wide range of linearity and needs only small amounts of plasmid DNA (50 ng).

3.4 Plasmid Stability during Storage and Application

Physical and chemical stability of plasmid DNA is a requirement for the development of DNA-based pharmaceuticals capable of being stored, shipped, and applied even under critical environmental conditions. DNA delivery sometimes requires the protection of this active pharmaceutical ingredient, and this is a DNA formulation issue. Guidance on the storage of plasmid DNA can be found in the ICH guideline "Stability testing of new drug substances and products" – Q1A (R2) of February 6th 2003 (ICH 2003).

3.4.1 Long-Term Stability of Plasmid DNA

The integrity and stability of DNA used in nonviral gene therapy is decisive for efficient gene transfer and transgene expression. The stability of the LacZ expressing plasmid pCMVβ stored at two different temperatures was monitored by CGE over a period of 13 months (Walther et al. 2003) and the data from this stability analysis were correlated with the *in vivo* transfer efficacy of plasmid DNA used in jet injection-based intratumoral DNA transfer.

Plasmid DNA was dissolved in water for injection at a concentration of 1 mg · mL^{-1} and the solutions were stored at –80 °C and at 4 °C. Initial quality control studies showed that 90% of the plasmid was in the desired ccc monomer form, 8% in the ccc dimer form, and 2% in the oc form. Plasmid homogeneity was analyzed over a period of 13 months: Figure 3.3 A–E shows a representative series of electropherograms. Figure 3.3 B and D represent the plasmid sample after storage at –80 °C for 1 and 13 months, showing that the distribution between ccc and oc forms is obviously unchanged.

A different result is observed for plasmid DNA stored at 4 °C, as indicated in Figure 3C and E. The fraction of ccc monomer and dimer is reduced and oc forms become prominent. After storage for 13 months another signal, representing the linear form of the plasmid, appears in the electropherogram, thus indicating degrading processes under these storage conditions. The plasmid homogeneity

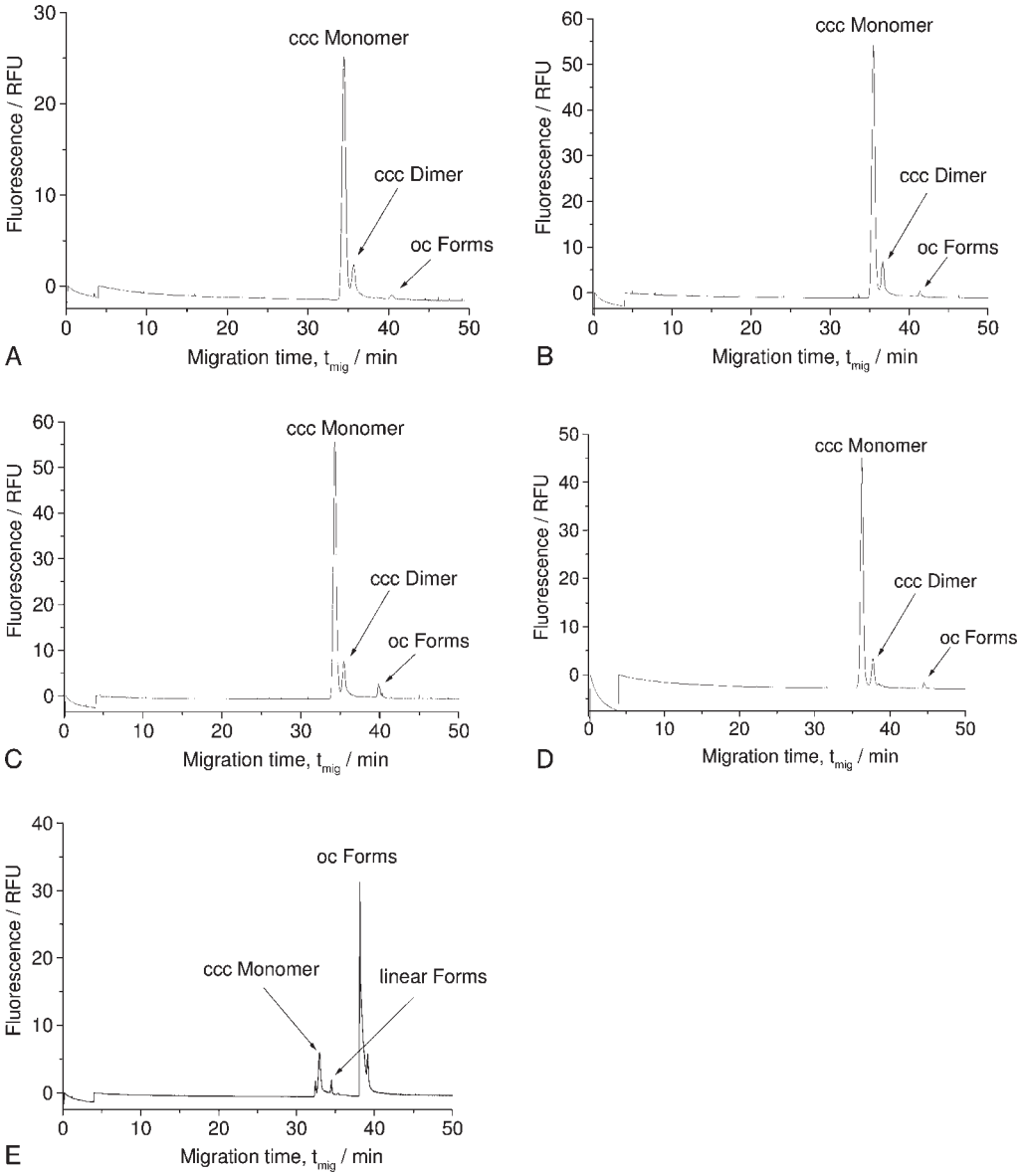

Figure 3.3 CGE analysis of storage conditions for plasmid pCMVβ at –80 °C and 4 °C after 1, 2 and 13 months. (A) Control material right after manufacturing. (B) Plasmid stored for 1 month at –80 °C or (C) at 4 °C. (D) Plasmid stored for 13 months at –80 °C or E) at 4 °C (from Schleef and Schmidt, 2004).

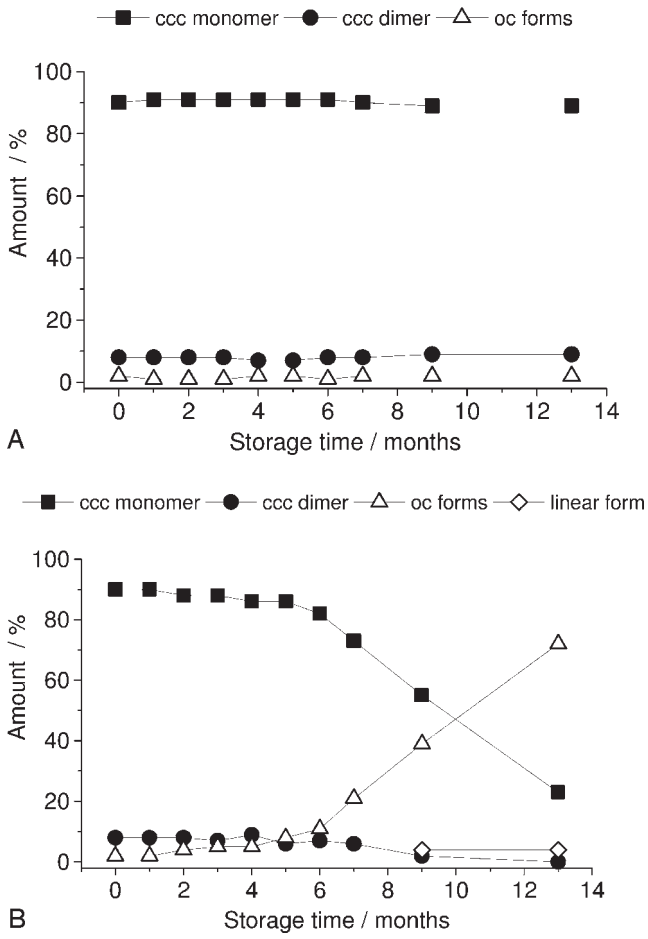

Figure 3.4 Quantitative analysis of plasmid DNA isoforms by CGE. Plasmid samples were stored at –80 °C (A) or 4 °C (B) over a period of 13 months and analyzed by CGE after 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 13 months of storage. Quantitative CGE data represent the percentages of the corresponding ccc and oc plasmid forms.

during storage is summarized in Figure 3.4: Figure 3.4 A represents the form size distribution at –80 °C, while Figure 3.4 B shows the distribution at 4 °C. Storage at –80 °C conserves the high amount of ccc monomer form and the low fractions of ccc dimer and oc forms. At 4 °C, degradation of plasmid DNA is observed after six months of storage, indicated by a decrease in the ccc monomer content and a corresponding increase in the oc forms. The data obtained here correlated with *in vivo* transfer efficiencies determined by jet injection, showing that suitable storage conditions not only stabilize the specific DNA conformation but also ensure reproducible results in *in vivo* gene transfer applications (Chapters 5 to 12).

3.4.2
Lyophilization for Long-Term Storage

Lyophilization (freeze-drying) is the most prominent technique for long-term conversation of biomolecules. The frozen product is dried under high vacuum, resulting in a nearly water-free, fluffy product, easily redissolvable in water or appropriate buffers. Generally, lyophilized products can be stored at room temperature for several years without negative influences on product quality. The major advantage of lyophilized products in comparison to those in aqueous solutions is that no expensive cooling chain with respective logistics is necessary for storage and shipment.

Since DNA is shear-sensitive, lyophilization of plasmid DNA products is not as easy as for other biomolecules. Extreme stressing of DNA in this process step generates single-strand breaks, resulting in increasing amounts of undesired open-circular forms. Figure 3.5 shows CGE electropherograms of plasmid DNA before and after lyophilization. No increase in the oc form amount can be observed after lyophilization, which makes this process step very suitable for plasmid DNA storage and shipment.

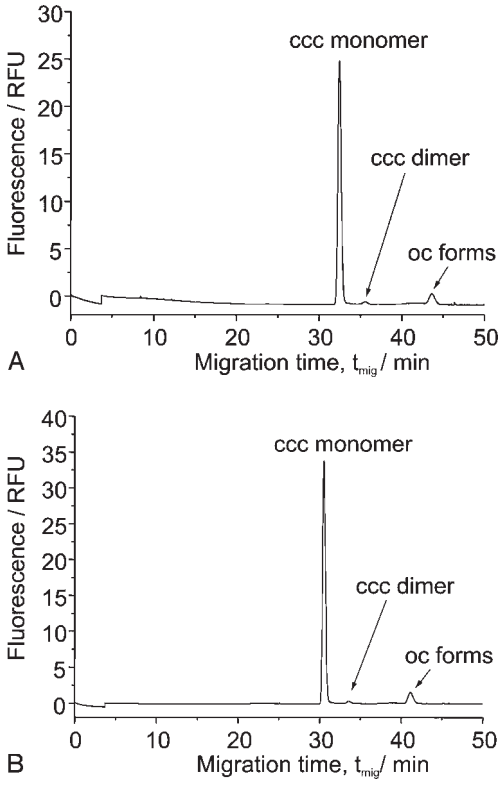

Figure 3.5 Analysis of plasmid DNA by capillary gel electrophoresis before and after lyophilization.

Table 3.4 Plasmid topology distribution of sample pFRef01, untreated pCMVβ sample and after jet injection of pCMVβ at different pressures (Walther et al. 2002).

Sample	*Sample description*	*ccc form*	*oc form*
Sample 2	DNA before filling into sample space	97.6%	2.4%
Sample 4	DNA from sample space after injection at 2.0 bar	93.5%	6.5%
Sample 5	DNA from sample space after injection at 2.5 bar	85.4%	14.6%
Sample 6	DNA from sample space after injection at 3.0 bar	81.6%	18.4%
Sample 7	Ejected DNA after injection at 2.0 bar	93.3%	6.7%
Sample 8	Ejected DNA after injection at 2.5 bar	84.6%	15.4%
Sample 9	Ejected DNA after injection at 3.0 bar	77.2%	22.8%

3.4.3
Stability during Application

Effective *in vivo* gene expression through persistent plasmid stability is dependent not only on storage conditions but also on the method for application of DNA drugs. Hydrodynamic methods (Chapter 9), gene guns (Chapter 10), or jet injection (Chapter 7) have been developed over recent years. Capillary gel electrophoretic analysis of plasmid homogeneity has also been applied to study the stability of plasmid DNA during application and to determine appropriate conditions (Walther et al. 2002).

Jet injection at pressures below 2.5 bar did not significantly degrade the ccc form of the sample (Table 3.4). However, injections above 2.5 bar showed a significant decrease, resulting in an increase in the oc form and probably in degraded forms. Jet injection at pressures below 2.5 bar, however, showed insufficient gene transfer into the tumor tissue. In consequence, appropriate conditions for efficient gene transfer have to be determined for different target tissue, with plasmid size and stability, as well as efficient penetration, being taken into account. Capillary gel electrophoresis has also proven itself as a valuable tool for this purpose.

3.5
Future Developments

Plasmid DNA in pharmaceutical development has so far been used to design the coding genes used for the production of therapeutic recombinant proteins. Potential further applications include the use of plasmid vectors for the production of viral particles, where plasmids may in some applications be part of the pharmaceutical. The direct application of DNA for prevention (vaccination) or gene therapy requires further development at different levels. Firstly, the vectors have to be more efficient (on the level of gene transfer and expression). Secondly, processes for large-scale purification of plasmid DNA at the multigram scale have to be developed.

Bottlenecks are found in the limitations of conventional chromatographic media. Operations such as extraction or affinity purification should certainly soon be competing with current state of the art methods.

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References

Barouch, D. H., Craiu, A., Kuroda, M. J., Schmitz, J. E., Zheng, X. X., Santra, S., Frost, J. D., Krivulka, G. R., Lifton, M. A., Crabbs, C. L., Heidecker, G., Perry, H. C., Davies, M.-E., Xie, H., Nickerson, C. E., Steenbeke, T. D., Lord, C. I., Montefiori, D. C., Strom, T. B., Shiver, J. W., Lewis, M. G., Letvin, N. L., Augmentation of immune responses to HIV-1 and simian immunodeficiency virus DNA vaccines by IL-2/Ig plasmid administration in rhesus monkeys. *Proc. Natl. Acad. Sci. USA* **2000a**, *97*, 4192–4197.

Barouch, D. H., Santra, S., Schmitz, J. E., Kuroda, M. J., Fu, T.-M., Wagner, W., Bilska, M., Craiu, A., Zheng, X. X., Krivulka, G. R., Beaudry, K., Lifton, M. A., Nickerson, C. E., Trigona, W. L., Punt, K., Freed, D. C., Guan, L., Dubey, S., Casimiro, D., Simon, A., Davies, M.-E., Chastain, M., Strom, T. B., Gelman, R. S., Montefiori, D. C., Lewis, M. G., Emini, E. A., Shiver, J. W., Letvin, N. L., Control of virimia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination. *Science* **2000b**, *290*, 486–492.

Birnboim, H. C., Doly, J., A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **1979**, *7*, 1513–1523.

Bridson, E., The development, manufacture and control of microbiological culture media. Basingstoke, UK: Unipath Ltd. **1994**.

Bussey, L., Adamson, R., Atchley, A., Methods for purifying nucleic acids. *WO 98/05673* (**1998**).

Check, E. (**2002**), Gene therapy: a tragic setback. *Nature* **420**, 116–118.

Colpan, M., Schorr, J., Moritz, P., Process for producing endotoxin-free or endotoxin-poor nucleic acids and/or oligonucleotides for gene therapy. *WO 95/21177* (**1995**).

Costioli, M. D., Fisch, I., Garret-Flaudy, F., Hilbrig, F., Freitag, R., DNA purification by triple-helix affinity precipitation. *Biotechnol. Bioeng.* **2003**, *81*, 535–545.

Doolan, D., Hoffman, S., DNA-based vaccines against malaria: status and promises of the multi-stage malaria DNA-vaccine operation. *Int. J. Parasitol.* **2001**, *31*, 753–762.

EMEA, Safety studies for gene therapy products, CPMP/SWP/112/98 draft, London **1998**.

EMEA, Note for guidance on minimising the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products, CPMP/410/01 rev 1, London **2001**.

Eon-Duval, A., Gumbs, K., Ellett, C., Precipitation of RNA impurities with high salt in a plasmid DNA purification process: use of experimental design to determine reaction conditions. *Biotechnol. Bioeng.* **2003**, *83*, 544–553.

Eon-Duval, A., MacDuff, R. H., Fisher, C. A., Harris, M. J., Brook, C., Removal of RNA impurities by tangential flow filtration in an RNase-free plasmid DNA purification process. *Anal. Biochem.* **2003**, *316*, 66–73.

FDA, Guidance for industry: Guidance for human somatic cell therapy and gene therapy. Rockville, MD: Center for Biologics Evaluation and Research, FDA **1998**.

Garner, M. M., Chrambach, A., Resolution of circular, nicked circular and linear DNA, 4 kb in length, by electrophoresis in polyacrylamide solutions. *Electrophoresis* **1992**, *13*, 176–178.

Ghose, S., Forde, G. M., Slater, N. K. H., Affinity adsorption of plasmid DNA. *Biotechnol. Prog.* **2004**, *20*, 841–850.

Goto, M., Ono, T., Horiuchi, A., Furusaki, S., Extraction of DNA by reversed micelles. *J. Chem. Eng. Japan* **1999**, *32*, 123–125.

Hatton, T. A., Reversed micellar extraction of proteins. In: Scamehorn, J. F., Harwell, J. H. (Eds.), *Surfactant-based separation processes*. New York, Basel: Marcel Dekker Inc. **1998**.

Horn, N., Budahazi, G., Marquet, M., Purification of plasmid DNA during column chromatography. *US 5707812* (**1998**).

Horn, U., Strittmatter, W., Krebber, A., Knüpfer, U., Kujau, M., Wenderoth, R., Müller, K., Matzku, S., Plückthun, A., Riesenberg, D., High volumetric yields of functional dimeric miniantibodies in *Escherichia coli*, using an optimized expression vector and high-cell-density fermentation under non-limited growth conditions. *Appl. Microbiol. Biotechnol.* **1996**, *46*, 524–532.

ICH harmonized tripartite guideline Q1A (2R), Stability testing of new drug substances and products, 6. February **2003**.

Johnson, P. H., Grossmann, L. I., Electrophoresis of DNA in agarose gels. Optimizing separations of conformational isomers of double- and single-stranded DNAs. *Biochemistry* **1977**, *16*, 4217–4225.

Lahijani, R., Hulley, G., Soriano, G., Horn, N. A., Marquet, M., High-yield production of pBR322-derived plasmids intended for human gene therapy by employing a temperature-controllable point mutation. *Hum. Gene Ther.* **1996**, *7*, 1971–1980.

Lander, R. J., Winters, M. A., Meacle, F. J., Buckland, B. C., Lee, A. L., Fractional precipitation of plasmid DNA from lysate by CTAB. *Biotechnol. Bioeng.* **2002**, *79*, 776–784.

Lee, A. L., Sagar, S., A method for large scale plasmid purification. *WO 96/36706* (**1999**).

Lee, S. Y., Chang, H. N., High cell density cultivation of *Escherichia coli* using sucrose as a carbon source. *Biotechnol. Lett.* **1994**, *15*, 971–974.

Lemmens, R., Olsson, U., Nyhammar, T., Stadler, J., Supercoiled plasmid DNA: selective purification by thiophilic/aromatic adsorption. *J. Chromatogr. B* **2003**, *784*, 291–300.

Levy, M. S., O'Kennedy R. D., Ayazi-Shamlou, P., Dunnill, P., Biochemical engineering approaches to the challenges of producing pure plasmid DNA. *Trends Biotechnol.* **2000**, *18*, 296–305.

Ljunglöf, A., Bergvall, P., Bhikhabhai, R., Hjorth, R., Direct visualisation of plasmid DNA in individual chromatography adsorbent particles by confocal scanning laser microscopy. *J. Chromatogr. A* **1999**, *844*, 129–135.

Macaloney, G., Hall, J. W., Rollins, M. J., Draper, I., Anderson, K. B., Preston, J., Thompson, B. G., McNeil, B., The utility and performance of near-infrared spectroscopy in simultaneous monitoring multiple components in a high cell density recombinant *Escherichia coli* production process. *Bioproc. Eng.* **1997**, *17*, 157–167.

Major, M. E., Vitvitski, L., Mink, M. A., Schleef, M., Whalen, R. G., Trépo, C., Inchauspé, G., DNA based immunisation using chimeric vectors for the induction of immune responses against the hepatitis C virus nucleocapsid. *J. Virology* **1995**, *69*, 5798–5805.

Mascola, J., Nabel, G., Vaccines for the prevention of HIV-1 disease. *Curr. Opin. Immunol.* **2001**, *13*, 489–495.

Michel, M.-L., Davis, H. L., Schleef, M., Mancini, M., Tiollais, P., Whalen, R. G., DNA-mediated immunization to the hepatitis B surface antigen in mice: Aspects of the humoral response mimic hepatitis B viral infection in humans. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 5307–5311.

Mountain, A., Gene therapy: the first decade. *Trends Biotechnol.* **2000**, *18*, 119–128.

Murphy, J. C., Wibbenmeyer, J. A., Fax, G. E., Willson, R. C., Purification of plasmid DNA using selective precipitation by compaction agents. *Nature Biotechnol.* **1999**, *17*, 822–823.

Nakano, K., Rischke, M., Sato, S., Märkl, H., Influence of acetic acid on the growth of *Escherichia coli* K12 during high-cell-density cultivation in a dialysis reactor. *Appl. Microbiol. Biotechnol.* **1997**, *48*, 597–601.

Paalme, T., Tiisma, K., Kahru, A., Vanatalu, K., Vilu, R., Glucose-limited fed-batch cultivation of *Escherichia coli* with computer-controlled fixed growth rate. *Biotechnol. Bioeng.* **1990**, *35*, 312–319.

Raper, S. E., Yudkoff M., Chirmule, N., Gao, G.-P., Nunes, F., Haskal, Z. J., Furth, E. E., Propert, K. J., Robinson, M. B., Magosin, S., Simoes, H., Speicher, L., Hughes, J., Tazelaar, J., Wivel, N. A., Wilson, J. M., Batshaw, M. L., A pilot study of *in vivo* liver directed gene transfer with Adenoviral vector in partial ornitine transcarbamylase deficiency. *Human Gene Ther.* **2002**, *13*, 163–175.

Reinikainen, P., Korpela, K., Nissinen, V., Olkku, J., Söderlund, H., Markkanen, P., *Escherichia coli* plasmid production in fermenter. *Biotechnol. Bioeng.* **1989**, *33*, 386–393.

Ribeiro, S. C., Monteiro, G. A., Cabral, J. M. S., Prazeres, D. M. F., Isolation of plasmid DNA from cell lysates by aqueous two-phase systems. *Biotechnol. Bioeng.* **2002**, *78*, 376–384.

Schleef, M. (Ed.), *Plasmids for therapy and vaccination*. Weinheim: Wiley-VCH **2001**.

Schleef, M., Schmidt, T., Animal-free production of ccc-supercoiled plasmids for research and clinical applications. *J. Gene Med.* **2004**, *6*, S45–S53.

Schluep, T., Cooney, C. L., Purification of plasmids by triplex interaction. *Nucleic Acids Res.* **1998**, *26*, 4524–4528.

Schmidt, T., Friehs, K., Flaschel, E., Rapid determination of plasmid copy number. *J. Biotechnol.* **1996**, *49*, 219–229.

Schmidt, T., Friehs, K., Flaschel, E., Structures of plasmid DNA. In: Schleef, M. (Ed.), *Plasmids for therapy and vaccination*. Weinheim: Wiley-VCH **2001**, 29–42.

Schmidt, T., Friehs, K., Schleef, M., Voss, C., and Flaschel, E. (**1999a**), Quantitative analysis of plasmid forms by agarose and capillary gel electrophoresis. *Anal. Biochem.* **274**, 235–240.

Schmidt, T., Schleef, M., Friehs, K., Flaschel, E. (**1999b**), Hochzelldichtefermentation zur Gewinnung von Plasmid-DNA für Gentherapie und genetische Impfung. *BIOforum* **22**, 174–177.

Schorr, J., Moritz, P., Schleef, M., Production of plasmid DNA in industrial quantities according to cGMP guidelines. In: Lowrie, D. B., Whalen, R. G. (Eds.), *DNA Vaccines: Methods and Protocols*. Totowa, NJ: Humana Press **1999**, 11–21.

Schroeckh, V., Hartmann, M., Birch-Hirschfeld, E., Riesenberg, D., Improvement of recombinant gene-expression in *Escherichia coli* for glucose-controlled continuous and fed-batch cultures. *Appl. Microbiol. Biotechnol.* **1992**, *36*, 487–492.

Schumacher, I., Freitag, R., Hilbrig, F., Method for treating biomass for producing cell lysate containing plasmid DNA. *WO 02/057446 A2* (**2002**).

Serwer, P., Allen, J. A., Conformation of double-stranded DNA during agarose gel electrophoresis: Fractionation of linear and circular molecules with molecular weights between $3 \cdot 10^6$ and $25 \cdot 10^6$. *Biochemistry* **1984**, *23*, 922–927.

Shiver, J. W., Fu, T.-M., Chen, L., Casimiro, D. R., Davies, M.-E., Evans, R. K., Zhang, Z.-Q., Simon, A. J., Trigona, W. L., Dubey, S. A., Huang, L., Harris, V. A., Long, R. S., Liang, X., Handt, L., Schleif, W. A., Zhu, L., Freed, D. C., Persaud, N. V., Guan, L., Punt, K. S., Tang, A., Chen, M., Wilson, K. A., Collins, K. B., Heidecker, G. J., Rose Fernandez, V., Perry, H. C., Joyce, J. G., Grimm, K. M., Cook, J. C., Keller, P. M., Kresock, D. S., Mach, H., Troutman, R. D., Isopi, L. A., Williams, D. M., Xu, Z., Bohannon, K. E., Volkin, D. B., Montefiori, D. C., Miura, A.,

Krivulka, G. R., Lifton, M. A., Kuroda, M. J., Schmitz, J. E., Letvin, N. L., Caulfield, M. J., Bett, A. J., Youil, R., Kaslow, D. C., Emini, E., Replication incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. *Nature* **2002**, *415*, 331–335.

Sinden, R. R., DNA structure and function. San Diego, CA: Academic Press **1994**.

Smith III, G. J., Helf, M., Nesbet, C., Betita, H. A., Mek, J., Ferre, F., Fast and accurate method for quantitating *E.coli* host-cell DNA contamination in plasmid DNA preparations *BioTechniques* **1999**, *26*, 518–526.

Southern, E. M., Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **1975**, *98*, 503–517.

Stadler, J., Lemmens, R., Nyhammar, T., Plasmid DNA purification. *J. Gene Med.* **2004**, *6*, S54–S66.

Strancar, A., Podgornik, A., Barut, M., Necina, R., Short monlithic columns as stationary phases for biochromatography. *Adv. Biochem. Eng. Biotechnol.* **2002**, *76*, 49–85.

Voss, C., Innovative Prozessstrategien zur Herstellung von Plasmid-DNA als Wirkstoff. *BIOforum* **2004**, *9*, 38–39.

Voss, C., Schmidt, T., Schleef, M., Friehs, K., Flaschel, E., Effect of ammonium chloride on plasmid DNA production in high cell density batch culture for biopharmaceutical use. *J. Chem. Technol. Biotechnol.* **2004**, *79*, 57–62.

Walther, W., Stein, U., Fichtner, I., Voss, C., Schmidt, T., Schleef, M., Nellessen, T., Schlag, P. M., Intratumoral low volume jet-injection for efficient nonviral gene transfer. *Mol. Biotechnol.* **2002**, *21*, 105–115.

Walther, W., Stein, U., Voss, C., Schmidt, T., Schleef, M., Schlag, P. M., Stability analysis for long-term storage of naked DNA: impact on nonviral *in vivo* gene transfer. *Anal. Biochem.* **2003**, *318*, 230–235.

Wan, N. C., McNeilly, D. S., Christopher, C. W., Method for lysing cells. *US 5837529* (**1998**).

Wang, F., Lee, S. Y., High cell density culture of metabolically engineered *Escherichia coli* for the production of poly(3-hydroxybutyrate) in a defined medium. *Biotechniol. Bioeng.* **1998**, *58*, 325–328.

Wolff, J. A., Malone, R. W., Williams, P., Chong, W., Acsadi, G., Jani, A., Felgner, P. L., Direct gene transfer into mouse muscle *in vivo*. *Science* **1990**, *247*, 1465–1468.

Woodgate, J., Palfrey, D., Nagel, D. A., Hine, A. V., Slater, N. K. H., Protein-mediated isolation of plasmid-DNA by a zinc finger-glutathione S-transferase affinity linker. *Biotechnol. Bioeng.* **2002**, *79*, 450–456.

4
Minimized, CpG-Depleted, and Methylated DNA Vectors: Towards Perfection in Nonviral Gene Therapy

Oleg Tolmachov, Richard Harbottle, Brian Bigger, and Charles Coutelle

4.1
Introduction

Gene therapy aims to achieve curative effects whilst avoiding unwanted side effects of the delivered vector system. In nonviral gene therapy approaches the delivered vector is often bacterial plasmid DNA, which can be easily produced in large quantity (reviewed in [1]). In addition to its therapeutic cargo, such a plasmid necessarily contains a bacterially derived replication origin and a selection marker, often a gene conferring antibiotic resistance. After production of the therapeutic plasmid DNA these sequences are no longer necessary, and are in fact detrimental to the therapeutic aim. Bacterial vector backbones mediate transgene silencing [2, 3] and induce inflammation in mammals through their 'CpG motifs' containing unmethylated C [4].

CpG dinucleotides (CpGs) constitute a core of somewhat longer sequences known as 'CpG motifs', present in bacterial DNA with the frequency that would be expected with random distribution of nucleotides for the given GC/AT ratio. In contrast, mammalian DNA in general contains CpG dinucleotides with a lower frequency than would be expected for random distribution (this is known as 'CpG suppression'). CpGs remain unmethylated in prokaryotic hosts such as *Escherichia coli*, and the mammalian immune system has been tuned by evolution to recognize the CpG motifs as foreign antigens and to mount an inflammatory response [5]. Enzymatic methylation of gene delivery vectors by CpG methylase (M.SssI, for example) has been employed to resolve the immune problems [3, 6, 7]. However, this approach is not as straightforward as it seems, because the rules that govern mammalian gene expression are complex. Mammalian CpGs are themselves only partially methylated, and an increased level of methylation (hypermethylation) is implicated in gene silencing. Thus, CpG methylation of plasmid DNA *in vitro* requires fine gauging of the extent of the methylation. The attention of many investigators has therefore been drawn towards the removal or sequence modification of the bacterial vector backbone and the ensuing reduction of the CpG load.

Here we discuss the hurdles to plasmid-based gene delivery presented by the mammalian immune system and review the various strategies intended to neutralize the side effects of the bacterial plasmid backbone. Removal of bacterial sequences and generation of a minimized DNA vector is the most universal and straightforward approach. An additional advantage of the minimized vectors is that the number of therapeutic sequences per unit weight of DNA is increased, thus enhancing transgene expression after transfer into a target cell. This aspect is particularly important if the amount of the administered DNA is limited by the toxicity of a DNA–polycation complex. Alternatively, vector minimization provides space for the addition of functional sequences to enhance nuclear import or to promote integration into the host genome. Concomitantly with minimization, the DNA vector can be transformed into a topological form more suitable for efficient transfection, although the choice of the best DNA form (see Chapter 3) for gene delivery is still controversial. The benefits of the minimized DNA vectors may come at a price, because the required additional biochemical manipulations can reduce DNA yield and compromise DNA purity, so in certain situations it is advantageous to use alternative DNA vectors with low immunogenicity, such as CpG-depleted and CpG-free plasmids, produced by standard plasmid DNA purification protocols.

4.2
The Mammalian Immune System as a Barrier to Nonviral Gene Delivery

Gene therapy vectors have to have 'stealth' properties in order to slip through a regime of immune surveillance in humans. The mammalian immune system has evolved a highly complex series of mechanistic, adaptive, and innate responses to invasions by pathogens, and bacterial DNA is a well known inducer of the innate responses. The resultant inflammation can provoke an adaptive response to plasmid-encoded products. The transgene products are recognized as foreign antigens by antigen receptors on the surfaces of B and T cells, and the ensuing clonal expansion and production of antibodies can eliminate the transgene-expressing cells.

The innate responses are evolutionarily ancient and are present in a much wider array of eukaryotes (reviewed in [8]). These responses are invoked by molecular structures present in pathogens but not in self-tissues, such as double-stranded RNA, lipopolysaccharide (LPS), and CpG motifs in double- or single-stranded DNA (reviewed in [9]). Cells of the innate immune system, such as plasmacytoid dendritic cells, natural killer cells, macrophages, and some B cells, have to be activated in order to generate effective responses. As most of these cells lack the specific receptors of T and B cells, they instead rely on pattern recognition receptors known in mammals as the Toll-like receptors (TLRs) (reviewed in [10]). To date, 12 Toll-like receptors have been identified, each with specificity for different molecular structures. It has been found that CpG-containing bacterial genomic DNA, plasmid DNA, and artificial CpG-oligodeoxynucleotides exert strong immunostimulatory effects through activation of TLR-9 [11]. Increased cytokine production can easily be detected after administration of DNA with unmethylated CpG motifs. In

particular, CpG-mediated stimulation of dendritic cells results in the production of IL-12, producing a potent inflammatory response (reviewed in [12]).

It is noteworthy that another major immunogen from Gram-negative bacteria, LPS, is recognized through TLR-4, and not TLR-9. Accordingly, DNA containing CpG motifs and LPS induces different spectra of cytokines [13]. Unlike TLR-4, which is displayed on the cell surface, TLR-9 has been shown to have an intracellular localization in a macrophage cell line [14]. This and other studies suggest that recognition of CpG motifs occurs in the endosomal compartment and may point at CpG internalization being an important step in immune stimulation.

A large body of work has focused on the identification of a consensus CpG motif for immune stimulation; this consists of 5′-XCGY-3′, where X is any base but C, and Y is any base but G [15]. In vertebrate genomes this consensus is a rare occurrence and CpG is methylated at the 5-positions of about 70% of the cytosines in mammals [16]. The optimal CpG motif for activating human immune responses is 5′-GTCGTT-3′, whilst the strongest immunostimulatory motif for activating mouse cells is 5′-GACGTT-3′ [4]. It should be noted that different CpG motifs can raise quite different immunostimulatory responses. Thus, so-called 'CpG-A-type' sequences induce copious amounts of interferon-alpha (IFN-α) and IFN-β, whereas so-called 'CpG-B-type' sequences induce maturation of plasmacytoid dendritic cells and elicit dramatically enhanced B cell proliferation [17].

One more issue to consider is the presence of unknown methylation-independent immunostimulatory motifs in plasmid DNA. The hypothesized existence of such motifs is supported by experiments that show a reduced, but still tangible, immune response to CpG-methylated plasmid DNA [18].

4.3
Strategies to Minimize DNA Vectors

To alleviate its deleterious properties, it is possible to remove the bacterial vector backbone entirely. A number of strategies based on propagation of plasmid DNA in bacteria and subsequent excision of the eukaryotic expression cassette from the plasmid by use of restriction endonucleases or by 'looping-out' through site-specific recombination have been devised in order to eliminate the bacterially derived vector backbone. Alternatively the therapeutic vector can be produced by using a bacterial origin of replication that has been maximally shortened and CpG-depleted by successive rounds of mutagenesis. A very short bacterial marker gene, such as *supE*, can be used for selection in bacteria. Finally, the minimized vector can be produced by PCR *in vitro*.

4.3.1 Excision of a DNA Fragment Containing a Transgene Expression Cassette from Plasmid DNA

Bacterial sequences can be cut out from the plasmid DNA by digestion with suitable restriction enzymes. As conventional DNA fragment isolation procedures are difficult to scale up, a minimized vector production procedure involving protection of the excised transgene expression cassette by terminal short hairpin DNA loops and subsequent degradation of the bacterial backbone by the exonuclease activity of T7 DNA polymerase was employed by the Schmidt–Wolf group [19]. The resultant minimized vector was called MIDGE and was shown to have reduced immunostimulatory activity.

Transfected DNA in mammalian cells is often found in the form of concatemers and there is evidence indicating that DNA concatemers are important for long-term transgene expression [20]. Notably, linear fragments gave higher activity of the transgene after Ca phosphate transfection of NIH 3T3 fibroblasts when they were ligated *in vitro* to produce concatemers [21]. As the terminal DNA loops of the MIDGE vector are likely to inhibit concatemerization, one can expect a reduced longevity of transgene expression after gene delivery with the MIDGE vector than after gene delivery with a simple linear DNA fragment.

There is no universal rule on whether to circularize and to introduce supercoiling into the excised DNA fragments containing the transgene expression cassette. Supercoiled plasmid DNA has been shown to provide superior efficiency of transfection in a number of tissue culture studies [22, 23], whilst another report showed more efficient mouse liver transfection by cleaved plasmid DNA than by the supercoiled plasmid DNA [2]. Interestingly, longevity of transgene expression was increased when restriction enzyme cleavage separated the transgene expression cassette and the bacterial backbone, indicating *cis*- but not *trans*-inhibition of longevity of transgene expression by the plasmid vector backbone [2].

4.3.2 Intramolecular Site-Specific Recombination Within a Bacterial Plasmid

The supercoiled state of DNA is usually retained after intramolecular site-specific recombination, so various site-specific recombination systems have been exploited to produce minimized plasmids in the covalently closed circular (ccc) form. A producer plasmid typically contains a therapeutic or a marker module flanked by recombination sites with their cores arranged as a direct repeat. Intramolecular recombination between these sites results in the generation of a 'miniplasmid' molecule containing the bacterial vector backbone sequences and a nonreplicating 'minicircle' molecule containing the therapeutic or marker module. As *in vitro* treatment of plasmid by a recombinase is difficult to scale up, the recombination is usually performed *in vivo* in the bacterial host [24, 25]. Expression of site-specific recombinase is induced after the bacterial culture harboring the minicircle producer plasmid has been expanded. Recombination products are then isolated by the

alkaline lysis procedure and minicircle DNA is purified. Normally the miniplasmid and remaining producer plasmid are linearized by *in vitro* restriction digestion with an endonuclease for which no recognition site is present in the minicircle sequence, and the covalently closed circular form of the minicircle is then isolated by ultracentrifugation in a CsCl density gradient in the presence of propidium iodide [25].

Weight for weight, minicircle DNA was 13 to 50 times more active than the corresponding plasmid DNA in marker gene transfer experiments into mouse muscle and into human tumors engrafted into mice [26]. The tail vein injection mouse model was used to show the superiority of the minicircle DNA, by a factor of 45 to 650, for Factor IX gene transfer into mouse liver [27].

Premature expression of a recombinase gene during the growth of the bacterial culture can result in the loss of the nonreplicating minicircle and in accumulation of the replicating miniplasmid, so tight control of the recombinase gene expression is crucially important. The temperature-sensitive *CI857* repressor/operator system of bacteriophage λ [24] and the *araC-araBAD* regulon [25], respectively, are sufficiently tight. Provided that adequate recombinase activity is achieved after induction, a single copy of the recombinase gene located on the bacterial chromosome is preferable, because it can help to ensure a virtually complete absence of the recombinase in the OFF state.

Site-specific recombination systems employed so far include the bacteriophage λ integrase/DNA topoisomerase IV complex catalyzing recombination between λ *attP* and *attB* sites [24], the *Streptomyces* bacteriophage ϕC31 integrase catalyzing recombination between corresponding *attP* and *attB* sites [27], and *Cre* recombinase, which catalyses recombination between *loxP* sites [25]. For a high minicircle yield, an irreversible recombination, in which no reentry of the minicircle into the recombination reaction takes place, presents a clear advantage. In the case of the λ integrase recombination system, such unidirectional recombination is achieved by employment of the *Xis*-deficient *E. coli* lysogen [24]. Similarly, the ϕC31 prophage excision function is absent in the *E. coli* host in the ϕC31 integrase-driven minicircle production system [27]. In contrast, *Cre* recombination is reversible. However, an equilibrium shift towards minicircle product can be achieved by employment of mutant *loxP71* and *loxP66* sites, which can efficiently recombine to produce the functionally impaired hybrid *loxP71/66* on the minicircle molecule and wild-type *loxP* on the miniplasmid molecule [25, 28].

All the currently existing minicircle DNA production systems can still be refined. Notably, a hybrid recombination site remains on the minicircle molecule as a last vestige of prokaryotic DNA and in theory could contribute to adverse reactions in mammals. The bacteriophage λ *attP* site is relatively bulky, so the resultant minicircle has over 250 bp of *attR* or *attL* sequences with a number of CpG dinucleotides, which can contribute to an inflammatory response if they remain unmethylated. In contrast, the *attP* site of bacteriophage ϕC31 is only 39 bp and the corresponding *attB* is 34 bp. However, the *attB* of ϕC31 contains four CpG dinucleotides, not surprising in view of the very high CG/AT ratio in *Streptomyces*. The bacteriophage P1 *loxP* site also contains two CpGs. While these facts are disturbing for a perfectionist, they are not too significant in a general context,

because expression cassettes for eukaryotic therapeutic or marker genes also often contain substantial numbers of CpG dinucleotides. While not all of these CpGs necessarily form CpG motifs that are recognized by the immune system in the unmethylated state, some of them do.

Some therapeutic cassettes are quite large (full-size dystrophin cDNA, for example, is over 11 kb). The sheer size of a multicopy plasmid necessary for minicircle production can compromise its structural and/or maintenance stability, so plasmid stability is a particular concern in minicircle DNA production. Good vector/insert combinations are often found empirically (O. Tolmachov, unpublished). Introduction of stabilizing functions from wild-type multicopy plasmids to minicircle producer plasmids should also be considered. Multicopy plasmids are known to be more stably maintained if supplied with a dimer-resolution system to maximize the number of independently segregating molecules and thus minimize the frequency of plasmid loss [29]. In this respect, the site-specific recombination system from plasmid RK2 used by Kreiss et al. [30] looks attractive. However, the focus of these authors was on production of monomeric minicircle DNA, so the *parABCDE'* locus of RK2 encoding the resolution machinery was added to the minicircle moiety of the minicircle producer plasmid, an addition that unfortunately increased the CpG load of the minicircle. As the benefits of monomeric minicircles over minicircle multimers are rather hypothetical, insertion of the DNA fragment that mediates dimer resolution (of the *parABCDE'* locus, for example) should be considered for the miniplasmid moiety rather than the minicircle moiety of the minicircle producer plasmid.

While alternative systems for minimized DNA production might eventually outcompete the minicircle strategy in some applications, one field of gene therapy seems to have an unavoidable requirement for minicircle DNA vectors. The 16.6 kb human mitochondrial genome codes for a number of functions and needs to be repaired or replaced in several hereditary diseases. The genome is tightly packed and unlikely to tolerate large inserts, so mitochondrial genome production in bacteria by the minicircle strategy is an attractive choice. The mouse mitochondrial genome has been produced as a minicircle DNA [25] and, once the human mitochondrial genome is cloned in *E. coli*, the technology should be applicable to generation of the full-size human mitochondrial genome minicircle.

4.3.3
Synthesis of Minimized DNA Vectors by PCR

An expression cassette without bacterial sequences can be generated by PCR through the use of a suitable recombinant plasmid as a template. The resultant PCR amplicon can be used directly as a gene transfer vector offering all the benefits of CpG reduction [31]. There are clear advantages in this strategy, including absence of contamination by bacterial LPS, a potent inducer of inflammation in mammals and thus a powerful inhibitor of gene transfer. In addition, direct vector generation by PCR can reduce the number of the DNA cloning steps (no construction of a minicircle producer plasmid is required, for example). The disadvantages of the

PCR production method include the introduction of a mutation load by Taq DNA polymerase and the high cost of proofreading thermostable DNA polymerases such as Pfu and Pfx. There are also limitations in terms of the size of the expression cassette amenable to PCR synthesis. Again, proprietary mixtures of Taq polymerase and proofreading enzymes known to perform well in long-range PCR are expensive. In addition, the potential side effects due to possible contamination of the PCR-generated therapeutic expression cassette by PCR primers should be carefully investigated.

4.3.4 Improvement of Minimized DNA Vector Yield and Purity

Gene therapy research on large animals and clinical trials require substantial amounts of pure vector DNA. Some steps in laboratory procedures for production of minimized vector DNA (such as ultracentrifugation in CsCl density gradients or PCR) are difficult to adapt to industrial scale. An additional challenge lies in improving the purities of the vector DNA preparations, as the quality of DNA from bacteria is often compromised by traces of bacterial LPSs, which tend to copurify with DNA. Even minor traces of LPS can be sufficient for the induction of inflammation in mammals, thus substantially reducing the efficiency of gene transfer. Some DNA purification steps (such as ultracentrifugation in CsCl density gradients in the presence of the intercalating dye propidium iodide) can introduce additional copurifying contaminants. Affinity chromatography is clearly a method of choice for minimized vector DNA production both in terms of its ability to cope with industrial scale processes and in terms of fine sequence-specific purification [32]. However, much laboratory work remains to be done to increase the yield of the minimized vector DNA and thus to enable a more economical industrial process.

Plasmid amplification, which capitalizes on the ability of ColE1-type plasmids to replicate after the inhibition of *de novo* bacterial protein synthesis with drugs such as chloramphenicol and spectinomycin, is often used to increase the yield of plasmid DNA [33]. Plasmid amplification can be of substantial advantage in minimized vector DNA production strategies based on excision of the therapeutic gene expression cassette from plasmid DNA. However, vector production strategies exploiting site-specific recombination are difficult to combine with plasmid amplification because inhibition of protein synthesis by antibiotics is normally irreversible and so expression of site-specific recombinase at the end of fermentation is impossible.

As the minimized vector DNA is generated in bacteria (with the exception of the PCR synthesis strategy), many aspects of its production can be manipulated through the bacterial genotype. LPS-depleted strains, for example, can be used to simplify DNA purification, general recombination deficient strains can be used to increase the structural stability of the minicircle-producing plasmids, and bacterially expressed inducible nucleases can be used to destroy miniplasmid DNA in order to simplify minicircle isolation. Indeed, it is possible to simplify removal of contaminating RNA from plasmid DNA preparations by employment of an engineered *E. coli* strain expressing RNAse A in the periplasmic space [34].

4.4 Depletion of CpG Dinucleotides in the Bacterial Vector Backbone

The laborious approach of gradual CpG dinucleotide reduction was pioneered by the Genzyme Corporation. Yew et al. [5] succeeded in a substantial depletion of CpG content in the minimal pMB1 plasmid origin of replication and kanamycin resistance gene by using successive rounds of site-directed mutagenesis and PCR-mediated assembly of single-stranded oligonucleotides. When standard, 'CpG-replete', plasmid DNA was compared to CpG-reduced plasmid DNA, the latter was shown to have a reduced toxicity and to confer a higher transgene expression level coupled with increased longevity of expression in immune-competent mice [35]. Interestingly, cleavage of the CpG-reduced plasmid to separate the expression cassette and the vector backbone did not enhance transgene expression, while the same procedure enhanced transgene expression several times if CpG-replete DNA was used [3]. This is a strong indication that inhibition of transgene expression is due to CpG dinucleotides in the bacterial moieties of the standard plasmid vectors.

At the time of writing it is possible to purchase (from InvivoGen) a plasmid vector completely devoid of CpG dinucleotides. The plasmid vector pCpG-LacZ consists of a mutant CpG-free version of R6K γ origin of replication, a CpG-free version of the bacterial EM7 promoter, a CpG-free version of the Zeo® resistance gene, a synthetic CpG-free mammalian promoter, a CpG-free allele of the *lacZ* gene, and a CpG-free form of the late SV40 polyadenylation signal. The eukaryotic moiety of the plasmid is insulated from the bacterial moiety by two MAR (matrix attached region) elements from the 5′ region of the human IFN-beta gene and beta-globin genes that are naturally devoid of CpGs. The plasmid can be propagated only in bacterial strains expressing the *pir* gene, which encodes the π protein that activates the R6K origin of replication.

4.5 Methylation of CpG Dinucleotides in Plasmid DNA

Plasmid DNA can be methylated to mimic the mammalian CpG methylation pattern and thus hide the CpG motifs from immune surveillance. The CpG-methyltransferase in common use is M.SssI from *Spiroplasma* sp. Methylation can be performed *in vitro* with a purified enzyme and *in vivo* in *E. coli* strains expressing M.SssI. Methylation *in vivo* appears to be more reproducible and complete [7]. However, mammalian CpGs are only partially methylated, and a hypermethylated status is in fact a hallmark of silenced genes (reviewed in [36]). Therefore, one might expect that blanket methylation of all CpGs in vector DNA might result in inhibition of transgene expression. Indeed, it was found that, although CpG methylation of plasmid DNA significantly reduced the inflammatory cytokine response, it also blocked expression of a number of marker genes [3, 6, 7, 37]. Surprisingly, expression of the CMV promoter-driven expression cassette for the CFTR gene was not inhibited, even though the CAT gene under the same promoter

was repressed [6]. This result might indicate the presence of neutralizing elements in the CFTR gene, irreproducible levels of methylation by M.SssI *in vitro*, or imperfections of the semiquantitative RT PCR assay for the CFTR gene expression used in this study. Clearly, the activity of M.SssI has to be carefully gauged to achieve an optimal combination of immune surveillance escape and high level of transgene expression.

4.6 Towards an Ideal Nonviral Vector

The fundamental problem remaining unresolved by current procedures for generation of therapeutic DNA in bacteria is the unmethylated status of CpG dinucleotides in therapeutic cassettes within mammalian DNA. While the bacterial vector backbone can be removed to produce minimized DNA vectors, the therapeutic module stays on, and some of its CpGs can be immunostimulatory. Mammalian DNA in general contains CpG dinucleotides with a reduced frequency, and not many of these CpGs can be expected to be present in the context of CpG motifs. However, the problem of residual immunogenicity of the minimized vectors can be pronounced if a therapeutic module contains a tight cluster of CpG dinucleotides known as a 'CpG island'. These regions are often associated with mammalian and viral promoters (such as CMV early promoter) and their methylation during differentiation constitutes an important mechanism of epigenetic regulation of gene expression (reviewed in [36]). As hypermethylation of the CpG islands is known to result in promoter shutdown, which is an undesirable outcome in gene therapy, blanket methylation of all the CpGs in vector DNA should be avoided. Perhaps the ideal solution is a combination of minimized DNA vector strategies with approaches involving partial CpG methylation either *in vitro* or in specially designed *E. coli* strains expressing suitable methyltransferases. In addition, one might consider addition of the 'neutralizing' sequences known to counteract the effect of unmethylated CpGs, such as $(5'\text{-TTAGGG-}3')_4$ [38] or 5′-TCCTGGCGGGGAAGT-3′ [39], to the minimized DNA vectors.

Minimized DNA vectors are attractive alternatives to viral gene delivery systems because of their low toxicity, relatively easy production, and great versatility. Like that of other nonviral vector systems, however, their efficiency is still below the requirements for realistic *in vivo* gene therapy. While minimization of the nonviral vectors allows one important obstacle in nonviral gene delivery – namely, immunotoxicity of plasmid DNA – to be addressed, there are still more hurdles, including the lack of inherent mechanisms for intracellular nuclear transfer and the only transient nature of gene expression, to overcome. There is therefore a need for the generation of novel minimized DNA vectors designed to be able to gain easy access to the nucleus by active intracellular import and to persist episomally, or subsequently to integrate into the host genome, thereby allowing sustained transgene expression. This may be accomplishable by introduction of recognition sequences into minimized vectors for specific binding of nuclear-transfer peptide signals [40]

and for genome integration or intranuclear maintenance of the eukaryotic expression cassettes.

4.7 Conclusion

Minimized DNA vectors are therapeutic or marker gene expression cassettes without unwanted bacterial plasmid backbones. They offer a number of advantages in nonviral gene therapy, most remarkably the reduction of the immunostimulatory CpG motifs in the vector DNA. A number of strategies to produce minimized DNA vectors have been devised, but large-scale production of pure minimized vector DNA is still a challenge. It may therefore be of benefit in a number of situations to use alternative weakly immunogenic DNA vectors, such as CpG-methylated, CpG-depleted, and CpG-free plasmids.

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References

1 Prather, K. J., Sagar, S., Murphy, J., Chartrain, M., *Enzyme Microb. Technol.* **2003**, *33*, 865–883.
2 Chen, Z. Y., He, C. Y., Meuse, L., Kay, M. A., *Gene Ther.* **2004**, *11*, 856–864.
3 Hodges, B. L., Taylor, K. M., Joseph, M. F., Bourgeois, S. A., Scheule, R. K., *Mol. Ther.* **2004**, *10*, 269–278.
4 Bauer, S., Kirschning, C. J., Hacker, J., Redecke, V., Hausmann, S., Akira, S., Wagner, H., Lipford, G. B., *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 9237–9242.
5 Yew, N. S., Zhao, H., Wu, I. H., Song, A., Tousignant, J. D., Przybylska, M., Cheng, S. H., *Mol. Ther.* **2000**, *1*, 255–262.
6 McLachlan, G., Stevenson, B. J., Davidson, D. J., Porteous, D. J., *Gene Ther.* **2000**, *7*, 384–392.
7 Reynes-Sandoval, A., Ertl, H. C. H, *Mol. Ther.* **2004**, *9*, 249–261.
8 Hoffmann, J. A., Kafatos, F. C., Janeway, C. A., Ezekowitz, R. A., *Science* **1999**, *284*, 1313–1318.
9 Takeda, K., Kaisho, T., Akira, S., *Annu. Rev. Immunol.* **2003**, *21*, 335–376.
10 Krieg, A. M., *Annu. Rev. Immunol.* **2002**, *20*, 709–760.
11 Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., Akira, S., *Nature* **2000**, *408*, 740–745. Erratum in: *Nature* **2001**, *409*, 646.
12 Wagner, H., *Adv. Immunol.* **1999**, *73*, 329–368.

13 Chow, J. C., Young, D. W., Golenbock, D. T., Christ, W. J., Gusovsky, F., *J. Biol. Chem.* **1999**, *274*, 10689–10692.

14 Ahmad-Nejad, P., Hacker, H., Rutz, M., Bauer, S., Vabulas, R. M., Wagner, H., *Eur. J. Immunol.* **2002**, *32*, 1958–1968.

15 Krieg, A. M., Yi, A. K., Matson, S., Waldschmidt, T. J., Bishop, G. A., Teasdale, R., Koretzky, G. A., Klinman, D. M., *Nature* **1995**, *374*, 546–549.

16 Ramsahoye, B. H., Biniszkiewicz, D., Lyko, F., Clark, V., Bird, A. P., Jaenisch, R., *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 5237–5242.

17 Rothenfusser, S., Hornung, V., Ayyoub, M., Britsch, S., Towarowski, A., Krug, A., Sarris, A., Lubenow, N., Speiser, D., Endres, S., Hartmann, G., *Blood*. **2004**, *103*, 2162–2169.

18 Cornelie, S., Poulain-Godefroy, O., Lund, C., Vendeville, C., Ban, E., Capron, M., Riveau, G., *Scand. J. Immunol.* **2004**, *59*, 143–151.

19 Schakowski, F., Gorschlüter, M., Junghans, C., Schroff, M., Buttgereit, P., Ziske, C., Schöttker, B., König-Merediz, S. A., Sauerbruch, T., Wittig, B., Schmidt-Wolf, I. G., *Mol. Ther.* **2001**, *3*, 793–800.

20 Chen, Z. Y., Yant, S. R., He, C. Y., Meuse, L., Shen, S., Kay, M. A., *Mol. Ther.* **2001**, *3*, 403–410.

21 Leahy, P., Carmichael, G. G., Rossomando, E. F., *Nucleic Acids Res.* **1997**, *25*, 449–450.

22 Weintraub, H., Cheng, P. F., Conrad, K., *Cell*. **1986**, *4*, 115–122.

23 Buttrick, P. M., Kass, A., Kitsis, R. N., Kaplan, M. L., Leinwand, L. A., *Circ. Res*. **1992**, *70*, 193–198.

24 Darquet, A.-M., Cameron, B., Wils, P., Scherman, D., Crouzet, J., *Gene Ther.* **1997**, *7*, 1341–1349.

25 Bigger, B. W., Tolmachov, O., Collombet, J.-M., Fragkos, M., Palaszweski, I., Coutelle, C., *J. Biol. Chem.* **2001**, *276*, 23018–23027.

26 Darquet, A.-M., Rangara, R., Kreiss, P., Schwartz, B., Naimi, S., Delaere, P., Crouzet, J., *Gene Ther.* **1999**, *6*, 209–218.

27 Chen, Z. Y., He, C. Y., Ehrhardt, A., Kay, M. A., *Mol. Ther*. **2003**, *8*, 495–500.

28 Albert, H., Dale, E. C., Lee, E., Ow, D. W., *Plant J*. **1995**, *7*, 649–659.

29 Tolmasky, M. E., Colloms, S., Blakely, G., Sherratt, D. J., *Microbiology.* **2000**, *146*, 581–589.

30 Kreiss, P., Cameron, B., Darquet, A. M., Scherman, D., Crouzet, J., *Appl. Microbiol. Biotechnol.* **1998**, *49*, 560–567.

31 Hofman, C. R., Dileo, J. P., Li, S., Huang, L., *Gene Ther.* **2001**, *8*, 71–74.

32 Costioli, M. D., Fisch, I., Garret-Flaudy, F., Hilbrig, F., Freitag, R., *Biotechnol. Bioeng.* **2003**, *81*, 535–545.

33 Frenkel, L., Bremer, H., *DNA* **1986**, *5*, 539–544.

34 Cooke, G. D., Cranenburgh, R. M., Hanak, J. A. J., Dunnill, P., Thatcher, D. R., Ward, J. M., *J. Biotechnology* **2001**, *85*, 297–304.

35 Yew, N. S., Zhao, H., Przybylska, M., Wu, I. H., Tousignant, J. D., Scheule, R. K., Cheng, S. H., *Mol. Ther.* **2002**, *5*, 731–738.

36 Meehan, R. R., *Semin. Cell. Dev. Biol.* **2003**, *14*, 53–65.

37 Yew, N. S., Wang, K. X., Przybylska, M., Bagley, R. G., Stedman, M., Marshall, J., Scheule, R. K., Cheng, S. H., *Hum. Gene Therapy.* **1999**, *10*, 223–234.

38 Gursel, I., Gursel, M., Yamada, H., Ishii, K. J., Takeshita, F., Klinman, D. M., *J. Immunol.* **2003**, *171*, 1393–1400.

39 Stunz, L. L., Lenert, P., Peckham, D., Yi, A. K., Haxhinasto, S., Chang, M., Krieg, A. M., Ashman, R. F., *Eur. J. Immunol.* **2002**, *32*, 1212–1222.

40 Vaysse, L., Harbottle, R., Bigger, B., Bergau, A., Tolmachov, O., Coutelle, C., *J. Biol. Chem.* **2004**, *279*, 5555–5564.

5
Localized Nucleic Acid Delivery: A Discussion of Selected Methods

Christian Plank, Franz Scherer, and Carsten Rudolph

5.1
Foreword

This book focuses on nucleic acid pharmaceuticals. The shuttles developed for nucleic acid delivery, so-called vectors, are either genetically modified viruses or synthetic constructs. Both vector types, if used for therapeutic purposes, can be regarded as formulations of nucleic acid pharmaceuticals. This chapter focuses on synthetic constructs for nucleic acid delivery, but also refers to viral vectors where appropriate. Localized delivery is an important objective for pharmaceuticals in general, including viral and nonviral shuttles for nucleic acid delivery. Before describing the aims and purposes of localized delivery we discuss general and mechanistic aspects of vectors and nucleic acid delivery, including references to the historical development of this research area. The selection of methods and ideas presented here is intended to provide an overview of current strategies without implying a valuation of individual strategies against one another; we do not, for example, discuss electroporation (Chapters 11 and 12), which has become one of the most powerful nucleic acid delivery techniques, in further detail, as this method has been discussed in excellent comprehensive recent reviews. It is our intent to highlight the importance of localization of delivery in general and the eminent role played by evolving physical techniques in this context.

Among the many methods and tools of localized delivery we therefore particularly focus on physical methods and discuss selected examples, including some of our own work, in more detail.

5.2
Nucleic Acid Delivery – What For?

Nucleic acids carry the building plans of living systems. Nucleic acid sequences are translated into structures and functions of cellular molecules, which, together with the biochemical reactions in which they participate, constitute the material aspect

of life. Located at the head of this cellular information flow, nucleic acids occupy a distinguished position among biological molecules. As the original information carriers, they participate, in an indirect manner, in any cellular process. Beyond their role as carriers and transmitters of information, nucleic acids also participate in a direct manner in cellular reactions. They have been known for a long time as structural and functional elements of multienzyme complexes such as ribosomes. In splicing reactions, for example, nucleic acids themselves carry some of the active ingredients of their own processing. In recent years it has become evident that nucleic acids participate directly in a multitude of cellular processes and thus contribute, maybe to an extent equal to that of proteins, to the coordinated and regulated network of cellular chemical reactions. RNA species (micro RNAs, short hairpin RNA, small interfering RNAs) in particular have been recognized as natural regulators of cellular processes [1, 2].

Given the distinguished role of nucleic acids in living systems, it is justified to conclude that any cellular process may be influenced to some particular purpose by the introduction of nucleic acids into cells from outside. Tatum formulated the basic concepts of gene therapy as early as in 1966, the year when the deciphering of the genetic code was concluded [3].

"Finally it can be anticipated that viruses will be effectively used for man's benefit in theoretical studies, in somatic cell genetics and possibly in genetic therapy. [...] We can even be somewhat optimistic on the long-range possibility of therapy by the isolation or design, synthesis and introduction of new genes into defective cells of particular organs. [...] We can be reasonably optimistic of the development, first, of effective preventive measures and, later, of curative therapy. These will come by epidemiological, immunological, and chemotherapeutic means, by modification and regulation of gene activities, or by means of gene repair and replacement. [...] Hence, it can be suggested that the first successful genetic engineering will be done with the patient's own cells, for example, liver cells, grown in culture. [...] The efficiency of this process and its potentialities may be considerably improved [...] by increasing the effectiveness of DNA uptake and integration by recipient cells."

Today's objectives of nucleic acid delivery – (1) complementation and over-expression of genes, (2) on/off regulation of genes, and (3) repair of genes – had already been formulated by this early stage. Tools for achieving nucleic acid delivery (viruses and DNA) had been designated. A major challenge of delivery, "increasing the effectiveness of DNA uptake and integration by recipient cells", had been defined. Six years later, Berg and colleagues provided experimental support for Tatum's visionary ideas by generating a recombinant SV40 virus that was able to transfer foreign nucleic acid sequences into mammalian cells [4]. A first major step had been taken towards the purposeful use of nucleic acids as research tools and as therapeutic agents.

5.3
Nucleic Acid Delivery – How?

It was believed for a long time that cells do not incorporate nucleic acids voluntarily, at least not in a manner that would result in the expression of an engulfed gene. Only relatively recently have we learned that, in certain cases, "naked" nucleic acids are efficiently taken up into cells in functional form ([5]; see further below). Before, there was agreement that shuttles for nucleic acid delivery would be required, as these polyelectrolyte macromolecules are unable to cross cellular membranes by passive mechanisms such as diffusion. Nature itself, however, has provided the ideal solution for this delivery problem in the form of viruses. These parasitic entities need to cross cellular membranes and ultimately need to shuttle their genetic information into cell nuclei in order to propagate. Consequently, genetically engineered viruses were among the earliest shuttles used for nucleic acid delivery and in many respects are still the most efficient. During the early days of manmade nucleic acid delivery, however, the modern tools of nucleic acid manipulation in the test tube were not available, so the construction of a genetically modified virus was a major challenge. In this respect it is less surprising that a nonviral chemical method, DEAE dextran precipitation (1967) [6], was in fact used earlier than viral vectors for nucleic acid delivery. A highly efficient method of nonviral delivery, calcium phosphate precipitation, still widely used today, was first described by Graham et al. in 1973 [7]. This method was an essential tool for the successful construction of adenoviral vectors. Retroviral vectors appeared in the early 1980s. In the meantime, a multitude of viral vectors have been described, each of them having its specific advantages and shortcomings. For nonviral vector engineers, it has been highly instructive to take a closer look at the major features of the naturally evolved solution to the nucleic acid delivery problem. The major functions of viral infectivity are as follows:

1. Viral genomes are packaged. Nucleic acids are compacted, such that the sizes of these macromolecules are compatible with the requirements of natural transport mechanisms. Packaging also protects the genome from degradation.
2. Receptor–ligand interactions. Viruses bind specifically to cell surface molecules, thereby gaining specificity in terms of host tropisms.
3. Exploitation of natural cellular uptake mechanisms such as endocytosis and mechanisms of escaping intracellular degradation. Many virus species enter cells through receptor-mediated endocytosis. The endosomal acidification process is exploited to trigger escape mechanisms, resulting in the release of the viral capsid from these internal vesicles.
4. Nuclear transport. Active transport across the nuclear membrane is exploited to localize viral genetic elements in the cell nucleus.
5. Genome organization. Viral genomes are organized in such a manner as to exploit the information storage capacity of nucleic acids in the most efficient ways (overlapping reading frames, bidirectional coding, differential splicing,

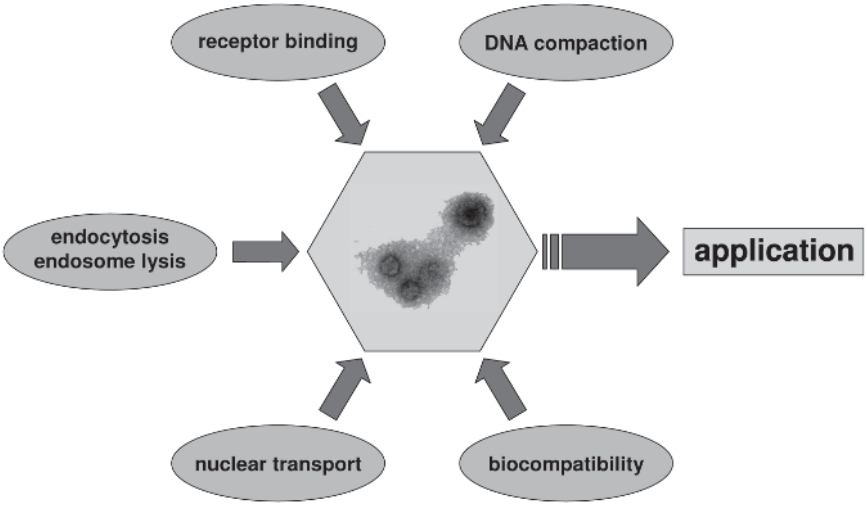


Figure 5.1 Nonviral vectors for nucleic acid delivery (sometimes called artificial viruses) are prepared by self-assembly of synthetic modules that mimic essential viral functions that allow them to infect cells. The self-assembly process is mostly based on noncovalent interactions of the individual modules, such as electrostatic and hydrophobic interactions. The most important interaction is that between the nucleic acid and a polycation or a cationic lipid, which can give rise to the formation of a charged nanoparticle that is able to transfect cells. The functionalities of receptor binding, membrane destabilization (such as endosome lysis), nuclear targeting, and biocompatibility can either be covalently coupled to a DNA binding/compacting moiety or can be incorporated into the complex as individual molecules by noncovalent interactions. The center of the figure shows toroidal nanoparticles typically formed upon mixing of plasmid DNA and polycations.

etc.). Furthermore, viral genomes are organized to exploit host functions, thereby minimizing the payload to be packaged in the viral particle.

6. Biocompatibility. Viruses are made up of natural materials. Although immunogenic, their constituents are biocompatible enough to warrant sufficient stability in the host during the extracellular phase of delivery to achieve target cell infection.

Nonviral vector engineers have mimicked these functions in creating synthetic modules that can be chemically or physically (self-)assembled to result in synthetic virus-like particles (often also referred to as artificial viruses; Figure 5.1).

5.3.1 Nucleic Acid Compaction

With examination of chromosome structure and function, it had been realized early on that DNA compaction is brought about by cationic sequences in histones and that such compaction can be achieved with synthetic oligo- and polycations.

The inventors of the DEAE/dextran and the calcium phosphate precipitation methods had found a physical way of DNA compaction. DNA packaging for delivery purposes has also been attempted by incorporation in the aqueous lumen of liposomes [8]. The extensive biophysical studies by many researchers on DNA compaction by cationic peptides [9–16] had obviously been forgotten when Wu and Wu [17, 18] and later on Wagner et al. [19] reported nonviral vector particles prepared from polylysine and plasmid DNA capable of transfecting cells *in vitro* and *in vivo*. The liposomal approach experienced a breakthrough when cationic lipids were first introduced as DNA binding and compacting agents [20, 21]. Important results of early nonviral vector research are that it is a natural property of nucleic acids as polyelectrolytes to "condense" into nanostructures upon mixing with polyelectrolytes of opposite charge (for further reading and review see [22–24]) and that the resulting complexes are able to transfect cells. As we now know, potency in cell transfection is not strictly dependent on DNA compaction. One important function of the cationic modules for DNA binding is that they mediate the binding of vector particles to cell surfaces in a nonspecific manner.

5.3.2
Receptor–Ligand Interactions

Nonviral receptor-mediated gene delivery was first introduced by Wu and Wu [17, 18, 25]. By coupling asialoorosomucoid, a natural ligand of the asialoglycoprotein receptor on liver cells, to the DNA-compacting moiety polylysine they generated vectors with increased target cell specificity that are taken up into cells by receptor-mediated endocytosis. Following a similar concept, Wagner et al. established *transferrinfection*, based on bioconjugates of transferrin and polycations that enter cells by transferrin receptor-mediated endocytosis [19, 26, 27]. In the meantime a multitude of suitable receptor ligands attached to nucleic acid binding moieties have been described. These include synthetic carbohydrates, synthetic peptides, recombinant proteins, immunoglobulins (antibodies), and other molecules such as folate. For recent reviews see [28–30].

5.3.3
Endocytosis and Endosomal Escape

Both the unspecific binding of vector particles to cell surfaces by electrostatic interaction and specific receptor–ligand-type binding result in endocytotic uptake into cells. This pathway subjects the internalized material to the cellular breakdown machinery in endosomes and lysosomes unless specific measures are taken to trigger endosomal escape. The required module has been provided both in biological and in chemical ways. Wagner et al. first described, and later refined, the use of pH-specific membrane-disrupting peptides for endosomal escape [31, 32]. Synthetic peptides with sequence analogy to the N-terminal sequence of the influenza virus hemagglutinin subunit 2 (HA-2) were chemically coupled to polylysine (the DNA compacting module) [31]. This sequence, capable of adopting

an amphipatic α-helix as its active conformation at acidic pH, is responsible for inducing the fusion of the viral and the endosomal membranes in the natural context. It is important to note that this sequence does not interfere with membrane integrity at neutral pH.

DNA complexes were formed by simple mixing of polylysine–INF peptide conjugates with plasmid DNA, optionally also containing a polylysine–receptor ligand conjugate as a cell surface binding module [33]. In a later refinement, INF peptides were incorporated into DNA complexes by electrostatic interaction [32]. Such complexes displayed greatly improved transfection efficiency in relation to standard polylysine–DNA complexes. In addition, the bee venom peptide melittin has also been used for endosomal release [34]. As this peptide also displays membrane-disrupting activity at neutral pH, suitable gene vector formulations and coupling strategies are required to minimize membrane disturbance by the vector as a whole at neutral pH and to maximize it at acidic pH (Ernst Wagner, personal communication). A breakthrough in terms of transfection efficiency was achieved when chemically inactivated adenovirus particles were coupled to polylysine–DNA complexes [35–37]. The genome of the virus was inactivated by psoralen treatment, which leaves the virus capsid and its endosome-disruptive function intact [38]. Coupled to an otherwise nonviral vector, this function highly efficiently mediates the release of the vector from endosomes.

Synthetic polymers on polyacrylic acid derivative basis with pH-specific membrane disruptive properties have been described [39, 40] and are useful in promoting drug and nucleic acid delivery across endosomal membranes [41–44].

Boussif et al. achieved endosomal escape based on the chemical structure of the DNA-compacting cationic moiety [45]. Polyethylenimine (PEI), a cationic chemical produced on industrial scales, binds and compacts DNA and by virtue of its secondary and tertiary amines has buffering capacity at physiological pH. In consequence, if a PEI–DNA particle is internalized into cells by endocytosis it will buffer the acidification process within endosomes. This means that the endosomal proton pump needs to pump far more protons into the endosome until the natural endosomal pH of about 5.5 to 6.5 is reached. The so-called "proton sponge hypothesis" postulates enhanced gene delivery due to the buffering capacity of polymers with structural features like those of PEI through enhanced endosomal chloride accumulation and consequent osmotic swelling/lysis. Sonawane et al. have provided experimental evidence supporting this hypothesis [46], directly measuring the previously postulated chloride accumulation and swelling of endosomes in living cells by elegant fluorescence techniques.

Additional mechanical destabilization may be provided through swelling of the internalized polymer itself, due to the electrostatic repulsion of its protonated amino groups. Earlier than PEI, polyamidoamine dendrimers were described as useful agents mediating gene delivery [47, 48]. Mechanisms similar to those in the case of PEI probably account for the activity of these polymers. A variety of other cationic polymers with protonatable amino groups have been described for nucleic acid delivery [49–58]. Some of them display reduced toxicity relative to PEI. Interesting alternatives to PEI also include poly(2-(dimethylamino)ethyl methacrylate) [59] and

biodegradable poly(2-(dimethylaminoethylamino) phosphazene) [60]. In terms of gene transfer efficiency, no single polymer outperforms the others to such a degree that it can be considered the polymer or lead structure of choice.

Polycation–DNA complexes are called polyplexes. The other major class of nonviral vectors are composed of cationic lipid formulations and nucleic acids. These are called lipoplexes. The endosomal escape of nucleic acids formulated as lipoplexes is thought to be mediated by lipid-exchange reactions between the endosomal membrane and the lipoplex (i.e., anionic lipids from the endosomal membrane compete with the nucleic acid for binding to the cationic lipid moieties and thereby release the nucleic acid from the complex), the endosomal membrane being destabilized through this process [61–63]. It is generally accepted that endocytosis is the major cellular uptake mechanism for lipoplexes. However, depending on the biophysical properties of lipoplexes, direct fusion with the cytoplasmic membrane can occur as well [64, 65]. Recent work by Safinya's group has resulted in an improved understanding of structure–function relationships in lipoplex-mediated nucleic acid delivery [65, 66]. The charge densities of lipid–DNA complexes are essential factors governing transfection efficiencies, at least if the lipids in the DNA complex are in lamellar configuration.

5.3.4 Nuclear Transport

It is still not well understood how and in what form nonviral vectors gain access to the nucleus. In any case, it is clear that the nuclear membrane represents a major barrier and bottleneck to gene delivery; in many cases, the breakdown of the nuclear membrane during cell division is a prerequisite for access to the nucleus. Nevertheless, the coupling of nuclear localization peptides directly to nucleic acids or the incorporation of such peptides into vector formulations has generated improvements to the delivery process. Background and recent progress in targeting to the cell nucleus is discussed in more detail later in this chapter.

5.3.5 Genome Organization

No major efforts have been invested in directly mimicking viral genome organization. Nevertheless, researchers have used viral genomic elements in order to enhance the persistence of transfected gene expression. Viral promoters such as the CMV promoter are widely used to drive the expression of a transfected gene. Plasmids that contain elements of Eppstein–Barr virus have been constructed in order to achieve extrachromosomal plasmid replication in eukaryotic cells (reviewed in [67, 68]). Elements from adeno-associated virus (AAV) responsible for the site-specific genomic integration of the virus have been used to generate a hybrid AAV–adenovirus vector carrying a double-reporter gene integration cassette flanked by AAV ITRs and a tightly regulated, drug-inducible Rep expression cassette [69]. Similar constructs can be delivered with nonviral technology. Site-specific genomic

integration has also been achieved with the ϕC31 integrase system. This is a recombinase found in a *Streptomyces* phage that mediates stable chromosomal integration of genes into host genomes without any additional cofactors [70]. The genomic integration is unidirectional and sequence-specific [71]. The ϕC31 integrase mediates the integration of *att*B attachment sites of the transgenic DNA into *att*P attachment sites in the host genome, which occur as pseudo-*att*P attachment sites in mammalian genomes [71].

5.3.6 Biocompatibility

Viruses are recognized as foreign by their host organism. Nevertheless, their constituents are biologic materials and viruses are biocompatible enough to achieve their replication in the host even though they may kill the host in doing so. From a biomaterial scientist's point of view, viruses are nanoparticles that are stable enough (biocompatible) during the delivery phase, yet their constituents are assembled in a manner labile enough to allow disassembly and biological processing once they have reached their target. It is not surprising that synthetic constructs for nucleic acid delivery are also recognized as foreign by the host organism. This recognition takes place on a systemic level during the extracellular delivery phase but also at the target cell level. First-generation nonviral vectors undergo strong interactions with blood components and are strong activators of the complement system [72]. These vector particles are mostly cleared from the systemic circulation by the reticulo–endothelial system. At the target cell level, the nucleic acid components of nonviral vectors may be recognized as foreign, one example being the interaction of unmethylated CpG sequences with toll-like receptor 9 (TLR9, see also Chapter 4) in intracellular compartments, initiating a signaling cascade resulting in the production of proinflammatory cytokines [73]. Another example is the induction of innate immune pathways by long double-stranded RNA, resulting in a generalized repression of protein synthesis [74].

Although no entirely satisfying solutions concerning the biocompatibility limitations to nonviral vectors are available, partial solutions have been provided. Inactivating interactions of vector particles with blood components can be reduced or even eliminated by appropriate surface modifications. These include the attachment of PEG chains, either covalently [75, 76] or noncovalently [77], or surface modifications by poly(acrylic acid) derivatives [39, 40] that are useful in promoting drug and nucleic acid delivery across endosomal membranes [41–44] or by *N*-(2-hydroxypropyl)methacrylamide [78]. The resulting vector nanoparticles are sterically stabilized, meaning that their interactions with each other and with third components are minimized by limiting the accessibility of their surfaces. Such surface modifications reduce the acute toxicities of vector particles, which can be lethal (in animal experiments; [79]).

In summary, considerable progress towards the construction of artificial virus-like systems for nucleic acid delivery has been made. Nonviral transfection has become an important tool in biological research and offers great potential in nucleic

acid therapies. Reagents for artificial vector construction made by the user approaching the efficiency of viral vectors are commercially available to anyone. Since the concepts of gene therapy were first formulated almost forty years ago, this field has experienced scientific breakthroughs, enthusiastic expectations, and serious setbacks. The validity and feasibility of the concepts have been demonstrated in thousands of animal experiments and in human clinical studies. Given the tremendous potential of nucleic acid-based therapies, the obvious question is why such therapies have not developed into widely practiced, state of the art treatments, at least in specialized hospitals, all over the world. The answer is that most current tools for the genetic modification of cells are still neither efficient enough or safe enough, nor are they affordable enough, simple to practice, or well understood. In consequence, similar limitations hold true for envisaged therapeutic strategies involving such tools. Nucleic acid delivery for therapeutic purposes is a highly complex challenge where multiple parameters can have a major impact on the therapeutic outcome. One such parameter is the ability to localize nucleic delivery.

5.4
Why is Localization of Drug and Nucleic Acid Delivery Important?

The maximum drug dose a patient can be given is that which he/she can ultimately tolerate, not the one that may be required to cure his/her disease. An instructive example is chemotherapy of cancer. Cytostatics have well defined potentials to kill cells in culture: a given dose will eradicate a given percentage of a cell population under consideration. In the patient, however, complex biodistribution patterns, drug metabolism, drug resistance, and the pharmacokinetics of a drug can limit its bioavailability at a target site. The patient is systemically "flooded" with a drug in order to achieve its threshold of action at the site of disease. Drugs are designed to act preferentially on selected biological processes in target cells, but absolute specificity in terms of target cell and target process is virtually impossible to achieve. Therefore, in the case of systemic administration of a drug, the threshold dose for target site action is often close to the threshold dose for undesired action at nontarget sites. In other words, the target-specific full dose–response range of a drug cannot be exploited to the level of saturation of the biologic process at which the drug is designed to act (Figure 5.2) [80]. Put yet another way, therapeutic windows of drugs are often narrow and undesired side effects are frequent. Therefore, localization (targeting) of drug delivery is an important objective and mainly serves three related purposes: firstly, to exceed the local threshold of drug action at the target site while remaining below this threshold at nontarget sites, secondly, to avoid side effects in this manner, and thirdly, to enlarge the therapeutic window (i.e., to exploit the full dose–response range of a drug locally).

A closer look at nucleic acid delivery highlights the importance of vector targeting and reveals that hierarchies of localization need to be discriminated. The probability of vector success (functional delivery of a nucleic acid to the desired subcellular localization) is the product of the probabilities of overcoming the individual barriers

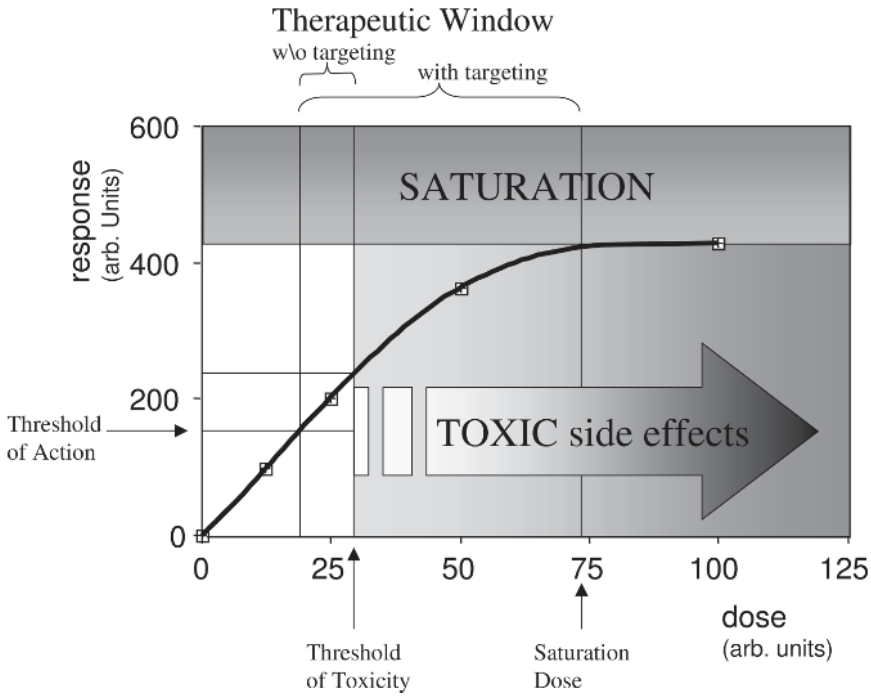


Figure 5.2 Toxic side effects often restrict the possibility of exploiting the full dose–response range of a drug up to (local) saturation levels. One objective of targeting to achieve target site saturation levels while pushing the non-target site toxicity threshold to higher doses. In this manner the therapeutic window widens enough to achieve a maximum local effect. Shown is a hypothetical dose–response relationship with arbitrary toxicity and saturation levels, just to illustrate the potential of drug targeting. (Reproduced from Plank et al. (**2003**) *Exp. Opin. Mol. Ther. 3(5)*, 745–758 [80]).

to delivery. These barriers may weigh differently on the final probability depending on vector type, but if the probability of vector-target cell contact is low to start with, the efficacy of the overall delivery process will be low as well, independent of vector type. Nonviral plasmid delivery with lipoplexes has been reported to be a mass action process [81], a statement that certainly also applies to other vector types (and drugs in general) if the frequency (or probability) of vector-target cell contact is a limiting barrier. For polyethylenimine(PEI)–DNA vectors it has been estimated that of about 700 000 plasmid copies applied per cell in a standard transfection, roughly 50 000 copies per cell will be present in the cell after 7 hours of incubation [82]. In another publication, it was estimated that one out of 100 microinjected cytoplasmic pDNA copies in a PEI–DNA formulation reaches the nucleus [83]. These two estimates together would predict that at least 1400 plasmid copies in PEI formulation per cell would be required in order to have one copy reach the nucleus. As it cannot be assumed that each cell-associated copy is located in the

cytoplasm, a more realistic estimate would predict rather that 10 000 or more copies in PEI formulation per cell would be required for this purpose. These estimates apply for one particular vector type in cell culture, where rapid vector inactivation, degradation, or clearance before it has a chance of target cell contact do not represent the major limiting barrier (although nonviral *in vitro* transfections are often carried out in serum-free medium to reduce vector inactivation). It is obvious that *in vivo*, where stability during the extracellular delivery phase represents a limiting factor, the required nucleic acid copy number per cell will be much higher than in cell culture. This applies to viral vectors as well.

So far, this discussion has focussed on a static view of dose–response relationships. Drug delivery, though, is a dynamic process in which residence times in individual compartments encountered during the delivery phase play an important role, especially if drug-inactivating interactions prevail in such compartments. Biologicals are particularly susceptible to inactivation and degradation, so the preservation of activity and delivery kinetics deserve particular attention. Methods for localized nucleic acid delivery often take account of the one or the other time-related aspect of drug delivery and drug action.

In summary, the threshold of action for nucleic acid delivery in terms of required copynumber per target cell can be quite high. Thresholds of action are related to the dynamics of delivery processes, to residence times in individual compartments along the delivery pathway, and to the physiological characteristics in, and to the boundaries between, such compartments. Methods for accumulating or holding an applied vector dose at a target site may be expected to improve the overall efficacy of nucleic acid drugs. It needs to be defined what target sites are and which measures may result in target site localization, and so it is useful to discriminate hierarchies of localization/targeting in terms of target characteristics on the length scale and in terms of processes required for reaching the target. Accordingly, methods of localization are discussed.

5.5
Hierarchies of Localization (Targeting)

"Localization" and "targeting" are used synonymously below. Useful classifications of drug targeting, exemplified by tumor targeting, have been published by Lübbe et al. [84] (Table 5.1). Among these, discrimination between first-, second-, and third-order targeting (Lübbe et al.) is useful, and in addition a forth order of targeting is appropriate for nucleic acid delivery. According to Lübbe et al., first-order targeting relates to the localization of a drug at the capillary bed of the target site (organ or tissue). Second-order targeting refers to the selective passage of the drug into tumor versus normal cells (generalized: target vs. normal), and third-order targeting involves uptake into cells by processes such as endocytosis. This classification sorts localization on a length scale and implies certain localization processes. By generalizing the classification of Lübbe et al., one can define hierarchies of localization.

Table 5.1 Hierarchies of localization in nucleic acid delivery.

Hierarchy	*Localization process*	*Delivery phase*	*Compartments/ constituents encountered*
I	Accumulation in target tissue versus systemic distribution	Extracellular delivery phase Administration site → target tissue	Systemic circulation Lymphatics Interstitium Blood (lymph) Components Extracellular matrix
II	Accumulation at/binding to target cells versus nontarget cells	Extracellular delivery phase Target tissue → target cells	Systemic circulation Lymphatics Interstitium Blood (lymph) Components Extracellular matrix Cell surface structures
III	Cellular uptake subcellular localization versus passive (random) distribution in target cell	Intracellular delivery phase Cell surface → intracellular localization	Endosomes, lysosomes, cytoplasm, nucleus, mitochondria
IV	Site-specific genomic integration versus random integration or extrachromosomal (episomal) localization	Subcellular delivery phase Nuclear localization → chromosomal integration	Cell nucleus

5.5.1 Methods of Localization and of Local Control

Many authors discriminate between active and passive targeting. The latter term refers to the preferred accumulation of a drug formulation or a gene vector in a particular tissue as a result of the biophysical properties of the formulation. Traditionally, active targeting is characterized as involving some form of molecular recognition that allows a formulation to interact specifically with target cells. This definition would mostly be limited to the biological methods of drug localization listed in Table 5.2. In a more comprehensive definition, modalities of active targeting not only comprise the provision of a formulation with a molecular recognition element but also any active procedure exerted on a formulation that will result in localized drug action. This would also include techniques for local *control* of delivery and nucleic acid expression, although such techniques do not qualify as methods of delivery in a strict sense. At least for nucleic acid delivery, it is useful to discriminate between biological and physical methods of localization. Both comprise various subtypes, which can often be combined in a flexible manner, including the combination of biological and physical subtypes. Most of the physical localization and drug activation methods listed in Table 5.2 would qualify as active targeting.

With respect to the hierarchies of localization listed in Table 5.1, most of these physical methods would serve hierarchy I, namely to accumulate a formulation in the target tissue. Biological methods of localization mostly serve hierarchies II–IV.

Table 5.2 Biological and physical methods of targeting in nucleic acid delivery.

Localization of delivery			
Biological	**Selected references[a)]**	**Physical**	**Selected references[a)]**
Receptor–ligand interaction	28, 30, 85	Passive targeting through biophysical properties of vector	86
Localization sequences	68, 87–90	Physical force used for vector accumulation	91
Site–specific genomic integration	67, 69, 92	• gravitational force – precipitate formation – centrifugation	6, 7, 93–98
		• magnetic fields	80, 99
		• hydrodynamic force (vector flow owards target cells, direct injection into target tissue)	100–105, Chapter 9
		• aerosolization	106, 107, Chapter 8
		• ballistic methods	91 and references therein, 108, Chapter 10
		• carrier-mediated (implants)	109, 110
		• injectable implants	111, 112
		• solid implants	110, 113–125
		• electric fields	91, 126, 127, Chapters 11, 12
Local control of delivery and expression			
Biological	**Selected references[a)]**	**Physical**	**Selected references[a)]**
Tissue-specific and inducible promoters ("transcriptional targeting")	128–130	Tissue-specific and inducible promoters ("transcriptional targeting" by electromagnetic radiation)	131–135
		Controlled release depots	111, 136
		Controlled release by electromagnetic radiation (heat)	136
		Ultrasound	137–140

[a)] Preferably review papers and not the primary literature are cited here.

Overcoming the cellular barriers to functional nucleic acid delivery (Table 5.2; localization hierarchies III and IV) is an ongoing challenge in vector construction. Synthetic modules for overcoming cellular barriers are described above and are continuously being improved. There is agreement that nuclear entry represents a major bottleneck to nonviral gene delivery, so we discuss nuclear localization in some detail. For better understanding of the various strategies to improve the nuclear delivery of DNA on which research has focused in recent years, a brief description of the mechanism of the cytoplasmic–nuclear transport mechanism of the mammalian cell is given.

5.5.2 Nuclear Transport of Macromolecules in Living Cells

The compartmentalization of the eukaryotic cell requires the import of all nuclear proteins from the cytoplasm into the nucleus and, vice versa, the export of all substances synthesized in the nucleus but required in the cytoplasm, such as transfer RNAs, messenger RNAs, and ribosomes. Nuclear import and export proceeds exclusively through the nuclear pore complex (NPC) by distinct pathways, including that by means of the large importin β-like nuclear transport receptor family. These receptors shuttle between the nucleus and the cytoplasm, thereby binding to the transport substrate either directly or through an adapter molecule such as importin α (classic import). The shuttling receptors all cooperate with the RanGTPase system, which is necessary to regulate their interaction with their cargoes (Figure 5.3) [141]. The NPC is composed of a large multiprotein structure of almost cylindrical appearance, measuring 125 nm in width and 150–200 nm in length and occurring in the nuclear membrane at a density of 1–10 NPCs $\cdot$ μm^{-2} [142]. The NPC forms an aqueous channel through which all of the transport proceeds, but the transport mode depends on the type of substrate transported through the NPC. Whereas small molecules such as metabolites pass the NPC by passive diffusion, the efficiency of this transport mode decreases as the molecular weight increases, due to the limited diameter (apparently 9 nm) of this transport channel. This theory finds evidence in the observation that proteins of a size of < 20–30 kDa diffuse relatively rapidly through the NPC, whereas bovine serum albumin (68 kDa, ~7 nm in diameter) diffuses through the NPC exceedingly slowly. The transport of large proteins into the nucleus thus requires an active and selective transport mode based on specific transport signals. The channel allowing such a transport mode opens to diameters of up to ~45 nm [143].

The nuclear transport receptors bind their transport cargoes in the cytoplasm through nuclear localization signal (NLSs) sequences and subsequently mediate their translocation to the nuclear side by direct interaction with the NPC, release the cargo, and finally return to the cytoplasm to begin a new shuttling cycle. The directionality of the transport process is accomplished through a RanGTP concentration gradient across the nuclear envelope (i.e., low cytoplasmic and high nuclear RanGTP concentration). RanGTP binds to the dimeric transport complex consisting of the nuclear transport receptor and the cargo in the nucleus, thereby

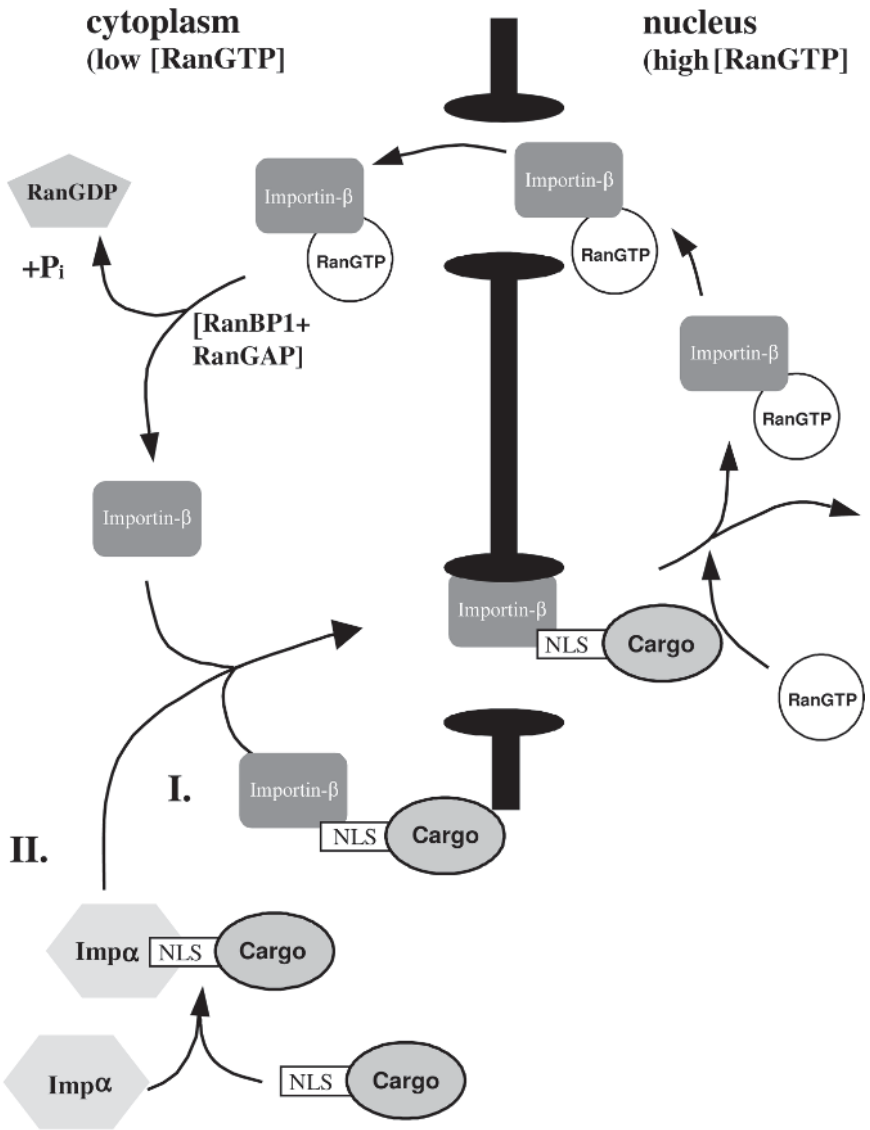


Figure 5.3 Schematic diagram of the transport signal mediated nuclear import (modified according to Göhrlich) [141]. (I) The transport substrate directly binds to the nuclear transport receptor of the importin β-family in the cytoplasm and proceeds through the NPC into the nucleus. In the nucleus, the transport substrate is released upon interaction with RanGTP and the nuclear transport receptor is recycled back into the cytoplasm (not illustrated in detail). (II) Importin α functions as an adapter molecule: binding of the transport substrate via a classical NLS and binding to the nuclear transport receptor importin β. The trimeric complex then proceeds into the nucleus, the transport substrate is released upon interaction with RanGTP, and the nuclear transporter is recycled back into the cytoplasm (not illustrated in detail).

dissociating the cargo from the nuclear transport receptor, resulting in the release of the cargo in the nucleus. In some cases the nuclear transport receptor does not bind directly to the transport substrate but requires an adapter molecule such as importin α (Figure 5.3 II) [141].

5.5.3 Nuclear Localization Signals and Gene Transfer

It was demonstrated earlier that only small DNA fragments (< 1 kb) are capable of traversing the nuclear pore energy-dependently, whilst large DNA fragments (> 1 kb) remain cytoplasmic upon cytoplasmic microinjection in living cells [81, 144] or upon application onto digitonin-permeabilized cells [145]. These observations resulted in the development of novel strategies to overcome the nuclear pore barrier, based on the naturally occurring nuclear localization signals (NLSs). NLSs are short peptide sequences predominantly made up of basic amino acids of endogenous or exogenous proteins such as transcription factors, ribosomal proteins, oncogene products, or the large T antigen of the simian virus [146], which mediate their transport from the cytoplasm into the cell nucleus by interaction with specific nuclear shuttle proteins (importin α or β, transportin) as described above.

Direct conjugation of 3–43 copies of a peptide comprising the NLS signal of the SV 40 large T-antigen (ACGAGPKKKRKV) to circular plasmid DNA resulted in specific, concentration-dependent binding to the nuclear shuttle protein importin α but transfection rates upon formulation with cationic lipids were significantly reduced by 60% as compared with unmodified plasmid DNA [147]. The authors suggested that direct modification of the plasmid DNA with a high number of peptides interferes with transcription efficiency. Very similar observations were reported by Sebestyen et al., who directly coupled up to 101 NLS per 1 kb of plasmid DNA (SV40 T antigen). Such coupling successfully induced nuclear import of plasmid DNA constructs in digitonin-permeabilized cells, but inhibition of transcription was observed [148]. Interestingly, no transfer of fluorescently labeled and peptide-modified circular plasmid DNA into the nucleus was observed when microinjected into the cytoplasm [147–149]. In a different approach, Zanta et al. coupled a single NLS of the SV40 large T antigen to the ends of hairpin-capped linearized plasmid DNA. Transfection of various cell types upon complexation with the cationic polymer PEI resulted in 10–1000-fold increases in gene expression, which was peptide sequence-dependent [150]. These observations indicate that receptor-mediated nuclear transport of plasmid DNA could be feasible under specific conditions. On the other hand, the findings of Zanta et al. are somewhat controversial [151].

Besides direct coupling of NLS peptides to plasmid DNA, various strategies have focused either on noncovalent incorporation of NLS peptides into gene vector complexes or on NLS modification of the gene transfer carrier. The addition of a peptide nucleic acid coupled to the SV40 NLS to plasmid DNA prior to complexation with PEI resulted in an eightfold increase in gene expression, which could be inhibited by an excess of free NLS [152]. Analogously, incorporation of a peptide comprising a nonclassical nuclear localization signal (NLS) containing the M9

sequence of heterogeneous nuclear ribonucleoprotein (hnRNP) A1 and a cationic peptide scaffold derived from a scrambled sequence of the SV40 T antigen consensus NLS in lipoplexes resulted in a 63-fold increase in reporter gene expression [153]. This effect was not observed for the scrambled M9 sequence, indicating a sequence-dependent mechanism.

Furthermore, the SV40 T antigen consensus NLS was coupled to linear and branched forms of the cationic polymer poly-L-lysine. The latter, known as loligomeres and each comprising a heptameric core of branched lysines conjugated to eight SV40 NLSs, demonstrated nuclear localization, but gene expression did not reach levels any higher than gene expression mediated by commercially available cationic lipids [154]. In contrast, coupling of 30–40 SV40 NLS peptides to a linear poly-L-lysine (MW 110 kDa) resulted in its selective binding to the nuclear shuttle protein importin α and nuclear accumulation in perforated cells both when the conjugate was complexed with plasmid DNA and when it was not. Transfected gene expression was doubled when the sequence of the SV40 NLS was used for conjugation but not with a transport-deficient mutant sequence [155].

In addition, complexation of plasmid DNA with the NLS of the large T antigen itself resulted in increased levels of nuclear translocation of cytoplasmically injected plasmid DNA [156]. In another approach, a tetrameric oligomer of the SV40 NLS (NLSV404) demonstrated to bind and compact plasmid DNA by electrostatic interaction and to form stable polyplexes was constructed [157]. The NLS404 peptide was capable of mediating sequence-specific nuclear accumulation of conjugated albumin and displayed nuclear transport properties for plasmid DNA, as confirmed by fluorescence *in situ* hybridization. Furthermore, NLSV404 polyplexes were shown to transfect various cell lines such as 16HBE14o-, HeLa S6, and Cos7 cells efficiently. NLSV404 polyplexes displayed transfection rates at least 20 times higher than those of analogous polyplexes formed by the nuclear transport-deficient mutant sequence cNLS. Combination of NLSV404 peptide with preformed polyethylenimine and dendrimer DNA complexes resulted in a strong increase in transfection efficiency. Incubation of cells with excess free peptide NLSV404, but not with a mutant control peptide, prior to transfection with NLSV404 polyplexes resulted in a dose-dependent decrease in the transfection rate, suggesting sequence-specific competitive inhibition. These results indicate that the NLSV404 was mediating nuclear accumulation of transfected plasmid DNA and that it can be a highly useful component of nonviral gene vectors.

An elegant fusion peptide containing both a membrane translocation domain (derived from the HIV gp41 fusion sequence) and the nuclear localization sequence of the SV40 large T antigen has been described by Morris and colleagues [158]. This peptide binds DNA by virtue of the cationic NLS sequence and promotes endocytosis-independent uptake of DNA into cells [159]. This potent delivery system, used to transfect a large panel of cell lines, has recently been used for nuclear targeting of siRNA directed against a promoter sequence in order to induce transcriptional gene silencing [160]. This peptide-based delivery system, called MPG by its inventors, is a particularly impressive example of how suitable vector engineering can be exploited to target nucleic acids to selected subcellular

localizations. While the parent MPG peptide interacts with the nuclear import machinery and targets nucleic acids to the nucleus, a peptide with a mutation of the NLS sequence can be used for rapid release of siRNA into the cytoplasm [159].

Oligomerization of a NLS peptide targeting the nuclear shuttle protein importin β, which represents a more direct strategy for targeting the nuclear import pathway, has been intensively studied [161]. In this study, multimers of the arginine-rich motif of the HIV-1 TAT protein (TAT peptide) were constructed and used as gene transfer carriers. The TAT peptide represents a NLS that mediates transport into the nucleus through importin β binding [162]. Conjugation of the TAT peptide with superparamagnetic nanoparticles [143], liposomes [163], and λ-phage [164] has been reported to result in their translocation into the nucleus. It was shown that oligomers of the TAT-(47–57) peptide compacted plasmid DNA into nanometric particles and stabilized plasmid DNA toward nuclease degradation. At optimized vector compositions, these peptides mediated gene delivery to cells in culture six to eight times more efficiently than poly-L-arginine or the mutant TAT(2)-M1. Precompaction of plasmid DNA with TAT peptides before addition of PEI, Superfect, or LipofectAMINE increased transfection rates by up to two orders of magnitude relative to the standard vectors. TAT-containing complexes transfected primary epithelial cells more efficiently and were superior to standard PEI vectors upon intratracheal instillation *in vivo*.

Interestingly, the NLSs used in all of these studies bind to different nuclear transport receptors such as importin α [150, 152, 156, 157], transportin [153], and importin β [161]. In conclusion, these results provide evidence that targeting of different nuclear transport receptors should in principle allow improvement of gene transfer efficiency of nonviral gene transfer systems.

As mentioned above, plasmid DNA (> 1 kb) remains in the cytoplasm (i.e., is excluded from the nucleus after cytoplasmic delivery) [81, 144]. In contrast with these findings, Dean et al. have reported that certain plasmid DNAs translocate into the nucleus after cytoplasmic delivery [165–167]. Such nuclear translocation has only been observed when a 72 bp fragment of the simian virus 40 (SV40) enhancer element is present on the plasmid DNA [166]. Interestingly, various transcription factor binding sites are located on the SV40 enhancer element. From this observation, the authors postulated a mechanism based on the characteristics of transcription factors to shuttle into the nucleus by exploiting the endogenous nucleocytoplasmic transport machinery [168].

As illustrated in Figure 5.4, the presence of the multiple transcription factor binding site within the 72 bp SV40 enhancer element results in the binding of the delivered plasmid DNA to newly synthesized transcription factors in the cytoplasm. These DNA binding proteins are normally located in the nucleus and contain NLSs that facilitate interaction with the nucleocytoplasmic transport machinery. The protein DNA complex is thus recognized via the NLS by a nucleocytoplasmic shuttle protein, thereby targeting the complex into the nucleus. This mechanism has been called "piggyback" transport.

In a more recent study, the transcription factor NFκB, which is activated by, for example, TNF-α, has been utilized to apply the concept of transcription factor-

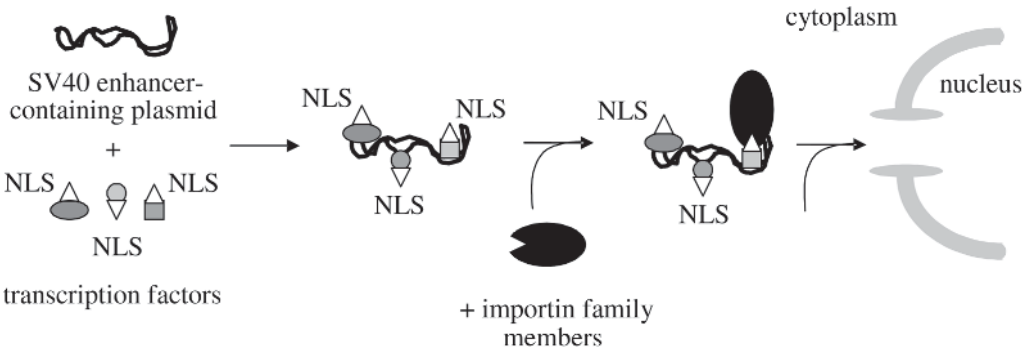


Figure 5.4 Mechanism of cytoplasmic-nuclear transport of plasmid DNA containing the SV40 enhancer element. Plasmid DNA containing the SV40 enhancer element binds to various transcription factors, thereupon mediating the interaction with a member of the importin shuttle protein family, resulting in the targeting of the plasmid DNA into the nucleus (modified according to Dean [166]).

mediated nuclear targeting of plasmid DNA. In this study, five direct repeats of the 11 bp Igκ κB motif, which binds with picomolar affinities to members of the NFκB/Rel family, were inserted downstream of a luciferase gene in a eukaryotic expression plasmid. The results of this study demonstrated TNF-α-inducible nuclear translocation of Igκ κB motif-containing plasmid, thereby resulting in NFκB-dependent transgene expression (35 times higher than with plasmid not containing the Igκ κB motif) [169]. The efficiency of this gene delivery system has further been applied in an *in vivo* approach. The intravenous injection of cationic lipid-based formulations (DOTMA/cholesterol) results in a transient inflammatory response in the lungs of mice, resulting in the activation of NFκB. Administration of a cationic lipid-based formulation comprising an Igκ κB motif-containing plasmid induced significantly higher gene expression in the lungs as compared with a control plasmid lacking the Igκ κB motif [170].

5.5.4
Localization Hierarchies I and II – Establishing Target Cell Contact

Equally important as overcoming cellular barriers to delivery is establishing vector–target cell contact in the first place. All downstream events are dependent on the frequency of this first step. Passive targeting based on the biophysical properties of vectors can be sufficient to achieve preferred transfection of certain tissues [86]; this is observed, for example, upon intravenous administration of PEI polyplexes [171] or of lipoplexes [172], resulting in high transfection levels in the lungs (Chapter 8) in mice. Interestingly, the pattern of transfection levels in the various organs does not match the actual biodistribution of the administered vectors. The major fraction of the applied vector dose is rapidly cleared by the reticulo–endothelial system [173], highlighting the importance of unspecific interactions *in vivo* and of

temporal aspects of delivery. Providing vectors with targeting ligands can greatly improve transfection efficiencies and specificities if nonspecific interactions can be reduced at the same time. This has been demonstrated *in vitro* and *in vivo*, particularly in tumor targeting upon intravenous administration. Vectors were shielded from nonspecific interactions by PEGylation, while targeting specificity was provided by epidermal growth factor (EGF) or transferrin [76, 174, 175]. Another example is a particular class of lipid-based nanoparticles with bound nucleic acids, provided with an $\alpha_v\beta_3$-targeting ligand, which mediated efficient and therapeutically relevant gene delivery to tumor endothelium [176].

Despite the encouraging success with targeted nucleic acid delivery in animal models, it is worth reconsidering the basic physics of the extracellular delivery phase from an administration site to the target cell surface. Cell culture serves as an instructive model from which conclusions for *in vivo* applications can be drawn. Luo and Saltzman have pointed out that DNA transfection efficiency is limited by a simple physical barrier: low DNA concentration at the cell surface [94]. Generalizing this observation, one can state that for a drug added to cell culture supernatants, drug–cell contact is driven by diffusion, no matter whether or not the drug carries a targeting ligand. As a first approximation, diffusion towards the target equals diffusion away from the target in the absence of binding or uptake events. The probability of cell–drug contact increases with drug concentration, incubation time, and temperature (which cannot be chosen arbitrarily), which explains why standard transfection procedures suggest over one hour of transfection time. In the presence of binding and uptake, the internalized drug amount should be proportional to some order of the drug concentration in the vicinity of the cell surface over a concentration range up to the saturation of the uptake process. The obvious prediction is that, below the saturation limit, any measure that increases the drug concentration at the target cell surface at a given drug dose will increase the response to the drug. Luo and Saltzman have verified this prediction for gene delivery and have substantiated it with theoretical analysis, by associating vectors with dense silica particles that sedimented vectors on the cell surfaces. Generalizing their observations, one can state that physical force acting on vectors directed in such a manner as to overcome motion away from the target enhances the delivery process. Suitable physical forces and delivery methods are listed in Table 5.2. The most convenient force for *in vitro* experiments is gravitation, as exploited by Luo and Saltzman and almost three decades earlier by Graham and Van der Eb in establishing the calcium phosphate precipitation method [7]. For PEI–DNA vectors, it has been found that large DNA complexes transfect more efficiently than smaller ones [93]. In fact, gravitation is exploited unwittingly by most researchers performing *in vitro* transfections with commercially available reagents. Most cationic lipids and polycations form precipitates with nucleic acids in salt-containing solution. Not surprisingly, centrifugal force also enhances nucleic acid delivery by accelerating vectors towards the cells to be transfected [95–98].

The options for drug administration *in vivo* are oral and parenteral. For obvious reasons, gravitation and centrifugation are not suitable for targeting in this case. Oral administration of gene vectors localizes delivery to the gastrointestinal tract

and offers great potential for genetic vaccination. Bacterial vectors [177–181], viral vectors [182, 183], chitosan–DNA complexes [184–186], and microencapsulated nucleic acids or viruses [187–193] are used for this purpose, and the reader is referred to the cited literature for details. A complete review volume has recently been dedicated to microencapsulated DNA formulations for vaccination purposes [194].

In parenteral administration, the choice is between local (orthotopic) and systemic routes. Success with biological vectors targeting receptor–ligand-type interactions upon systemic administration has already been briefly discussed. However, the above considerations for cell culture, in which diffusion has been defined as a limiting barrier, suggest that the probability of vector–target cell contact upon systemic administration will be even orders of magnitude lower than *in vitro*. Depending on the target tissue, the accessibility of target cells may be limited, diffusion may be restricted, and hydrodynamic forces (e.g., blood flow) may carry vectors away from the target site. In this respect it is particularly encouraging that site-specific transfection is possible even without further provisions for retention at the target. Nevertheless, the prediction holds (with restrictions) that any measure that increases the vector concentration at the target cell surface at a given administered dose will increase the response (e.g. level of transfected gene expression). The restrictions are that the applied measure must not interfere with vector integrity, uptake, and intracellular processing. Our own work with magnetic field-guided delivery confirms this prediction.

5.5.5
Vector Localization by Magnetic Force (Magnetofection)

We define magnetofection as nucleic acid delivery guided and mediated by magnetic force acting on associates of magnetic particles and nucleic acids (Figure 5.5). This comprises both "naked" nucleic acids and "packaged" nucleic acids, in which the packaging may be in the form of a synthetic nucleic acid vector, but may also be in the form of a virus.

We developed magnetofection [195] after learning about the concept of magnetic drug targeting. This concept is similar to the application of gravitational or centrifugal force, but in contrast, magnetic drug targeting is applicable *in vivo* for increasing the concentration of a drug formulation at the target cell surface. Drugs are associated with magnetically responsive materials in the nano- to micrometer size range and in that manner can be "navigated" by magnetic force. As early as the mid 1960s, researchers were attempting the first steps to produce magnetically localized thrombi in intracranial aneurisms, both in animals and in humans [196–199], through the use of carbonyl iron. Pioneering work by Widder and colleagues [200] inspired research into magnetically accumulating drugs, mostly in tumors, upon administration into the circulation. The magnetic carrier materials are mostly iron oxides of various compositions, which can be of natural or synthetic origin [80, 201–203]. Magnetic albumin microspheres with entrapped doxorubicin were magnetically accumulated in a Yoshida sarcoma in a rat model. A 100 times higher dose of free doxorubicin was required to achieve the same drug level as the

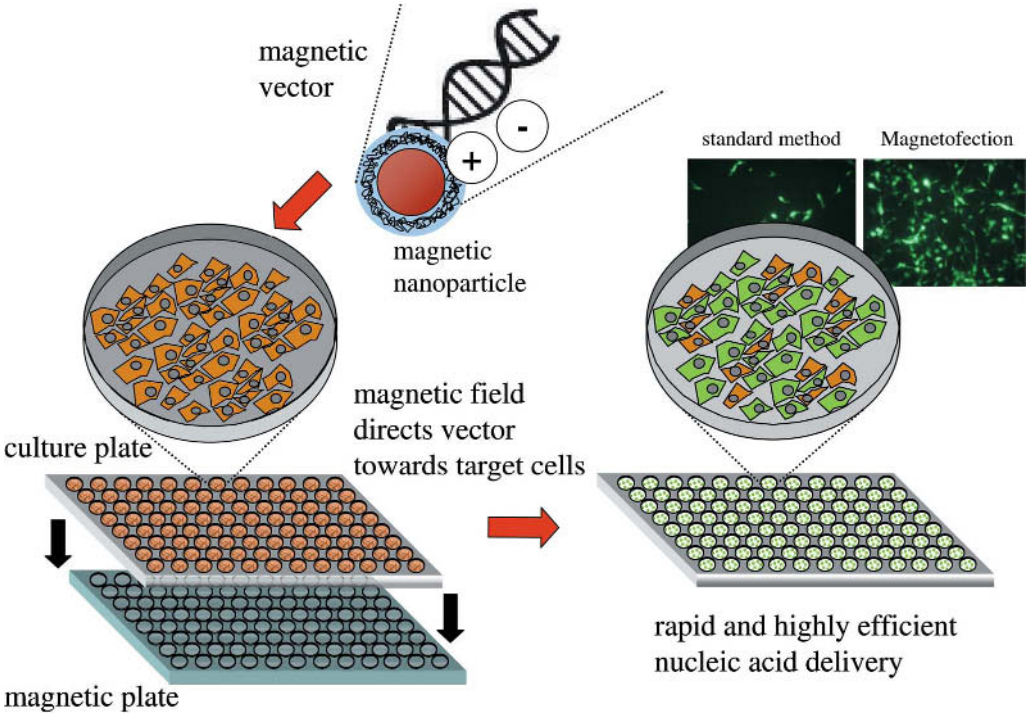


Figure 5.5 Principle of magnetofection in cell culture. Polyelectrolyte coated magnetic nanoparticles are mixed with naked nucleic acids or synthetic or viral nucleic acid vectors in salt-containing buffer. The particles associate with nucleic acids and vectors by electrostatic interaction and/or salt-induced colloid aggregation. The mixtures are added to cells in culture. The cell culture plate is positioned on a magnetic plate for 5 to 30 minutes of incubation. The magnetic field(s) rapidly sediment vectors on the cells to be transfected/transduced. The result is rapid kinetics and high efficiency nucleic acid delivery. Shown is a cell culture plate and a magnetic plate in 96-well format. The magnetic plate consists of 96 individual neodymium-iron-boron magnets (IBS Magnets, Berlin, Germany) inserted in drill holes in an acrylic glass or PVC plate in strictly alternating polarization. The plate was designed for application with 96-well cell culture plates but is also applicable for 24-, 12- and 6-well layouts, Petri dishes of various diameters, and culture flasks of various sizes. Detailed protocols can be found at www.ozbiosciences.com. (Reproduced from Schillinger et al. (**2005**), *J. Magn. Magn. Mat.* [234]).

magnetically targeted drug in the tumor [204]. The treatment was therapeutically effective in that it resulted in total tumor remission in a high percentage of experimental animals. In contrast, animals treated with free doxorubicin, placebo microspheres, or nonlocalized doxorubicin microspheres exhibited significant increases in tumor size with metastases and subsequent death in 90–100% of the animals [205, 206]. Other researchers obtained similar results [207–213]. After extensive preclinical examinations, Lübbe et al. applied magnetic drug targeting in cancer patients [214–217]. Retardation of tumor growth and even local remissions were observed [217]. A different type of magnetic particles (MTCs, Magnetic Targeted

Carriers) [218–221] are being used in another clinical study with magnetically targeted doxorubicin in which 32 patients have reportedly been enrolled [222]. In the meantime, however, a phase II/III clinical trial involving this technology has been discontinued as the clinical endpoints could not be met with statistical significance (http://freshnews.com/news/biotech-biomedical/article_17775.html).

This highlights the difficulties encountered when proceeding from animal to clinical studies, and hopefully a thorough failure analysis will be published at some point. Nevertheless, at least in animal models it has been clearly demonstrated that: (1) magnetic drug targeting is feasible even if the drug administration site is remote from the target site under magnetic field influence [84, 215], (2) the magnetic particles can extravasate under the influence of the magnetic field [209, 219, 223], and (3) the magnetic carriers are well tolerated.

Magnetic targeting of nucleic acid pharmaceuticals is in an early preclinical phase. It was necessary to associate nucleic acids or vectors with magnetic particles in a manner compatible with cellular uptake and the desired intracellular processing. Surprisingly, this has been a relatively simple task; we have used magnetic iron oxide nanoparticles coated with cationic or anionic polyelectrolytes for this purpose [80, 99, 195]. The natural tendency of charged colloidal particles to aggregate in salt-containing solution is usually considered an annoying characteristic because it limits the stability of colloidal suspensions under physiological conditions. The same problem applies for nonviral nucleic acid vectors, which are also charged nanoparticles. However, we used the otherwise undesired salt-induced aggregation to associate vectors with magnetic nanoparticles. Simple mixing of the vector components (polycation and/or lipid, nucleic acid or viruses) with polyelectrolyte-coated magnetic nanoparticles in salt-containing solution (such as cell culture media or physiological buffers) is sufficient to obtain the desired magnetic vectors. In our own work we have predominantly used polyethylenimine-coated iron oxide nanoparticles, but we have also shown that other polycationic and polyanionic surface coatings are suitable for magnetofection [80, 224]. Most recently, Haim et al. have used negatively charged magnetic nanoparticles coated with derivatized starch to associate these with lentivirus preparations [225] in a noncovalent manner. This is achieved, as the authors argue, by colloidal clustering facilitated by positively charged ions in solution [226]. Other researchers have used colloidally stable streptavidin-coated magnetic particles and biotinylated vectors for the same purpose [227–230]. In cell culture, these magnetic vectors can be sedimented on the cells to be transfected by magnetic fields within a few minutes, with the consequence that the full vector dose rapidly comes into contact with the target cells (the diffusion limitation is overcome). As predicted, this greatly improves the dose–response profiles of most examined gene vectors (an example is shown in Figure 5.6).

Incubation times can thus be limited to minutes instead of hours. We have shown that, at least in the case of antisense oligonucleotide delivery, the rapid transfection kinetics helps to reduce transfection-associated toxicity to the cells [231]. Another consequence of magnetically guided nucleic acid delivery is that it can be confined to cells under the influence of the magnetic field within one cell culture dish. We have discussed the details and benefits of the magnetofection method in several

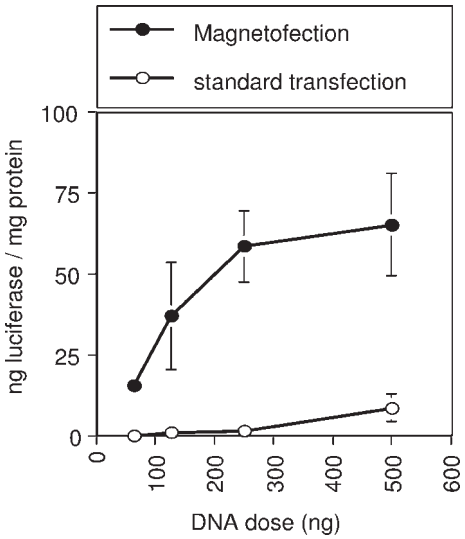


Figure 5.6 Standard transfection and magnetofection of B16F10 mouse melanoma cells. The figure shows typical dose–response relationships observed when comparing magnetofection and standard transfection. In this case, the cells were seeded in a 96-well plate at a density of 6000 cells per well on the day prior to transfection. For the standard transfection, plasmid DNA coding for luciferase was mixed with DOTAP-cholesterol liposomes (1 : 0.9 mol/mol) to result in a charge ratio of 1.25 (positive charges of DOTAP over negative charges of DNA). For magnetofection, DOTAP-cholesterol liposomes were mixed with DEAE dextran-coated magnetic iron oxide nanoparticles (obtained from Chemicell, Berlin, Germany) followed by mixing with plasmid DNA. The w/w ratio of magnetic particles to DNA was 2, the charge ratio of DOTAP-cholesterol to DNA was 1.25. After addition of the DNA complexes to the cells, the culture plate was positioned on a magnetic plate for 20 minutes (see Figure 5.5). Luciferase expression was determined 24 hours after transfection. The figure shows that saturation levels of transfection are achieved with magnetofection, while the standard reagent at the same dosage remains considerably below this level. To achieve the same effect with the standard reagent, high doses that would give rise to toxicity would be required (compare Figure 5.2).

publications and so will not repeat these here; the interested reader is referred to the primary literature [80, 98, 99, 195, 231–234]. Briefly summarized, the linkage between magnetic particles and vectors can be established in a reversible manner. Therefore, cells can obviously dissociate the components, and the association is compatible with the required intracellular processing steps. Magnetofection appears to be universally applicable to viral and nonviral vectors and among the latter to the delivery of large (plasmid DNA) and small synthetic nucleic acids (antisense oligonucleotides and siRNA [80, 231, 234]). Uptake into cells proceeds through endocytotic processes as for the parent vectors, and the applied magnetic force appears to have no further effect beyond localizing vectors at the target cell surface [98]. The only mechanistic differences between standard transfection and magnetofection observed so far were with adenoviral vectors and with siRNA delivery [80, 195, 234]. The association of adenovirus with cationic magnetic particles allows

the vector to infect cells that do not express the coxsackie and adenovirus receptor (CAR). Synthetic siRNA molecules cannot be delivered in a functional manner with linear PEI. If combined with cationic magnetic particles and magnetofected, however, otherwise inactive linear PEI–siRNA complexes efficiently knock down target gene expression [80, 234]. The mechanistic basis for this has not been elucidated so far. We have used magnetofection very successfully for the transfection of primary cells, including lung epithelial cells [233], blood vessel endothelial cells [232], keratinocytes, chondrocytes, osteoblasts, and amniocytes (unpublished results), as well as with whole tissue specimens of airways [233] and with blood vessels ([195, 231] and unpublished results). In the meantime, magnetofection reagents are commercially available from OZ Biosciences (Marseille, France. www.ozbiosciences.com) and Chemicell (Berlin, Germany. www.chemicell.com). Accordingly, more publications involving the method can be expected in the near future.

An important question is whether magnetic nucleic acid targeting is feasible *in vivo* and whether magnetofection is useful beyond research applications in nucleic acid-based therapies. We have provided proof of principle in demonstrating magnetically localized transfections in segments of blood vessels and in the gastrointestinal tract [195]. We have also demonstrated therapeutic potential in an ongoing veterinary clinical study of immuno gene therapy of feline fibrosarcoma [234]. This is one of the most common feline tumors, with a relapse rate of 75% within six months upon surgical resection, the standard therapy (see [235] for more details on feline fibrosarcoma). We inject a plasmid construct with the human GM-CSF gene under the control of the CMV promoter associated with magnetic particles directly into the tumor twice, with a one-week interval, starting two weeks prior to surgical resection of the tumor. During the application, a neodymium–iron–boron magnet is placed on the tumor adjacent to the injection site in order to retain the injected dose within the tumor tissue, so in this case, magnetic field guidance is not used to direct the vector to the target tissue upon remote administration but rather to keep a locally applied dose in the target tissue. The interim result of this study is that tumor-free survival of the cats is raised from only 23% at the one year time point in the case of standard therapy (surgery only) to 52% with presurgical magnetofection of the human GM-CSF gene (20 patients treated).

One can conclude that magnetically guided nucleic acid delivery has potential *in vivo*. At the same time, limitations are clearly evident, although some of these may be overcome by appropriate formulations and novel magnetic field technologies. Magnetic nanoparticles in a magnetic field move in a preferred direction of space only if they experience a magnetic field gradient. The magnetic force acting on a particle is proportional to the magnetic flux density, to the volume (and thus the third power of the radius) of the particle, and to the field gradient. During *in vivo* applications, hydrodynamic forces counteract magnetic retention. An example is the viscous drag force according to Stoke's law in the blood stream, which is proportional to the first power of the particle radius. Detailed theoretical considerations substantiated with experimental evidence have been published [236–239]. A study by Nagel [239] shows that magnetic particles with diameters in

the lower nanometer range (around 50 nm) are not suitable for magnetic drug targeting. In agreement with theoretical predictions, only a minor percentage of magnetic particles could be trapped with the use of rare earth permanent magnets even at low flow rates of up to 4 mm · s^{-1} as prevalent in small capillaries. Increasing of the particle diameters helps, but upper limits are set by the anatomy of blood vessels (capillary diameter of about 5 μm). Magnetic drug targeting appears impossible at flow rates around 20 cm · s^{-1}, such as in the human aorta. Another limitation is that magnetic flux density and field gradients decrease rapidly with increasing distance from a magnetic pole shoe. Gradients cannot be generated arbitrarily in space. Hence, for the moment, magnetic drug targeting is limited to superficial or surgically accessible areas of an organism. Nevertheless, even with the given constraints, numerous applications of magnetic targeting can be envisaged. Blood flow rates may be reduced locally and temporarily, the vasculature of major organs is accessible to catheters, strong electromagnets with tailored field gradients are being constructed, and suitable formulations containing magnetic particles developed. Nagel's study suggests that magnetic deposition of magnetic particles against hydrodynamic force is a cooperative process. Particles, once deposited, generate additional local field gradients in an external field, and these facilitate the deposition of further particles. Babincova et al. have suggested the positioning of ferromagnetic materials close to a target site [240]. In a strong external homogenous field, such as is present in magnetic resonance imaging equipment, such material will generate strong local gradients that may be exploitable for magnetic drug targeting. Similar ideas were presented at a recent meeting of the magnetic particle research community (see www.magneticmicrosphere.com for further information). Important developments can be expected in the near future, particularly if methods of active biological targeting are combined with passive targeting and physical force fields.

5.5.6
Hydrodynamic Methods of Nucleic Acid Delivery

In the previous section, hydrodynamic force, particularly in the bloodstream, was discussed as opposing magnetic targeting. Surprisingly it has been found that hydrodynamic force can itself be effectively exploited to achieve nucleic acid delivery (Chapter 9).

In the 1980s several groups had found that direct injection of plasmid DNA *in vivo* resulted in the expression of the encoded protein [241–243]. In 1990, Wolff and coworkers found that direct intramuscular injection of naked DNA and RNA expression vectors resulted in high and persistent transfected gene expression [5], and the Wolff group and other researchers confirmed this finding in numerous subsequent studies [104]. Budker et al. found that naked DNA injected in hypertonic solution intraportally in mice with transient occlusion of hepatic veins resulted in quite efficient gene delivery to hepatocytes [244], in a study later extended to injections of hyperosmotic DNA solutions into afferent and efferent hepatic vessels under transient occlusion of blood outflow in mice, rats, and dogs [245]. Extra-

ordinarily high levels of reporter gene expression were achieved and hepatocytes became transfected throughout the liver.

In 1999 it was shown in two independent studies that rapid injection of large volumes of DNA solutions into tail veins of mice resulted in enormous expression levels in the livers of the animals, with up to 40% of the hepatocytes becoming transfected [246, 247]. In these so-called hydrodynamic methods of nucleic acid delivery, volumes equaling or exceeding the actual blood volumes of the experimental animals are injected (see [102, 104, 105] for reviews). The mechanism of this method (in mice) involves, not surprisingly, a transient irregularity of heart function, but also, importantly, an enlargement of liver fenestrations and a transient permeabilization of hepatocyte membranes [248]. The authors also refer to the method as hydroporation. Hydrodynamic delivery has primarily been highly useful as a research tool. It allows evaluation of gene functions, assessment of therapeutic activities of genes and gene therapy concepts, or examination of siRNA-mediated expression knockdown *in vivo* [249–251]. Most recently, it was shown that the method may be relevant in therapy, as it can be applied in transiently isolated limbs to achieve highly efficient nucleic acid delivery throughout muscle cells of the isolated limb [252]. In contrast with preceding procedures [253], the administration of nucleic acids was performed via distal veins, a clinically viable procedure, rather than via arteries. The treatment was tolerated well in mice, rats, dogs, and nonhuman primates.

Hydrodynamic methods of nucleic acid delivery are a combination of orthotopic (localized) vector administration and an acceleration of vectors towards target cells with concomitant permeabilization of the target tissue.

5.5.7
Local Vector Implantation. Carrier-Mediated Nucleic Acid Delivery

Most methods of nucleic acid delivery involve carriers in one way or another. The term "vector" itself designates carrier materials, where the carrier material may be plain nucleic acid without further additions (naked plasmid DNA can be a carrier material of a gene to be delivered, for example). As discussed, nucleic acids are formulated with additional compounds that may function as pilots along the delivery pathway. So far, our considerations have been focused on vectors that are small in comparison with the target cells (nanometers to a few micrometers in diameter). From a different point of view, the term "vector" can comprise objects covering several orders of magnitude in diameter (nanometers to centimeters) if "vector" is regarded as one supply entity of nucleic acids. A vector, as an entity, can be large in comparison with the target cell.

The term "delivery" implies motion, and a provider and a customer. The "provider" is the vector, the "customer" is the target cell. Both can be either stationary or mobile. We have discussed vectors as the mobile elements in the delivery process upon which physical force can be exerted to "accelerate" them towards or into target cells. We have considered target cells as stationary and neglected the fact that cellular and intracellular motion is a key element at least during the final stages of delivery.

Cells move if provided with the appropriate signals and the appropriate scaffolds. This is a natural process during tissue development, comprising cell differentiation and dedifferentiation processes, cell maturation, and tissue regeneration (wound healing) in adult tissue and in malignant neoplasias. Biomaterials such as collagen, fibrin, or bone constituents are known as excellent scaffolds for cell growth. Researchers have developed synthetic materials – biomimetic, biodegradable, or stable but biocompatible – that can serve as matrices or surfaces for cell colonization. Such materials have been used successfully as implantable carriers for drugs [254] such as antibiotics [255], recombinant proteins (growth factors) [256] or nucleic acids [109, 113, 257]. These composites can be regarded as macroscopic vectors, which are moved towards target cells by physical force (implantation). Once in contact with the target cells, these can move into the supply depot and take up the microscopic constituents of the macroscopic vector. At the same time, the microscopic constituents may be released from the depot in a more or less controlled manner.

Prolonged and localized gene expression is desirable for the treatment of various inherited or acquired diseases. Besides the need for prolonged gene expression of therapeutic genes restricted to specific local tissues, local gene expression could also be used for vaccination purposes or for the treatment of inherited diseases such as hemophilia A and B. In these cases, localized delivery would not necessarily be required. Rather, the locally transfected cells function as bioreactors, producing the relevant gene product. Nevertheless, the prolonged character of gene expression is desirable in each of the applications. Prolonged gene expression has been successfully achieved by incorporation of plasmid DNA either into nanospheres and microspheres, or into scaffolds consisting of either synthetic or naturally occurring biodegradable polymers [258]. Such controlled release systems have been shown to increase gene expression and to enhance the duration of transgene expression relative to that achieved with naked plasmid DNA delivery upon injection of aqueous solutions. As an advantage of these systems, naked plasmid DNA or gene vectors are delivered locally, which avoids distribution to more distant tissues and reduces both toxicity to nontarget cells and immune response to the gene vector. Generally, the plasmid DNA is entrapped within the polymer matrix of the controlled release formulation and is released from these materials by a combination of diffusion and polymer degradation. The polymer might increase gene expression by plasmid DNA protection against microenvironmental enzymatic and non-enzymatic-induced degradation and maintains the plasmid DNA concentrations at effective doses. Depending on the type of polymer and its structure, the release kinetics of plasmid DNA from the polymer matrix can be controlled, resulting in sustained gene expression in the surrounding tissue.

Fang et al. were the first to explore and successfully to demonstrate the possibility of using collagen sponges as implantable carriers for naked plasmid DNA, coining the term “gene activated matrix” (GAM) for their technique [259]. The concept is to provide cells with a scaffold for growth, where they can pick up genetic information that, once expressed, will direct cell differentiation in an autocrine and paracrine manner (Figure 5.7). Cells are made to produce their own drugs locally. Obviously,

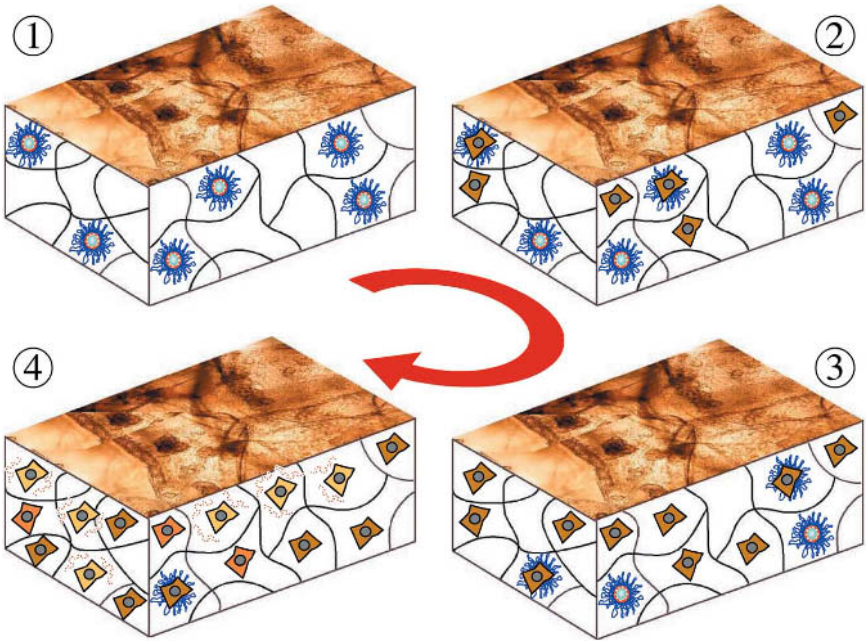


Figure 5.7 The gene-activated matrix concept schematically illustrated with copolymer-protected gene vector-loaded collagen sponges. Such sponges are prepared as described by Scherer et al. [113]. The sponges are soaked with a vector suspension and are freeze-dried ①. If such preparations are added to cells in culture or are implanted *in vivo*, cells start to colonize the sponges ②, and take up the immobilized gene vectors ③. This results in the expression of the gene encoded by the vector. In the case of growth factor genes, the expression product (the growth factor) will be secreted, resulting in autocrine and paracrine stimulation of cells colonizing the sponges, which can produce a desired cell differentiation process ④. Such preparations can be used to promote wound, cartilage, or bone healing or to promote local neoangiogenesis. The size relationships in Figure 5.7 do not correspond to the real situation. The vectors shown in this figure (*COPRPOGs*) are 20–30 nm in diameter.

apart from genetic vaccination, tissue engineering is an area where such concepts promise their strongest potentials. The question that arises is why should a nucleic acid therapy concept be chosen if the same or similar effects can be generated with recombinant growth factors? The answer is that their use is restricted in terms of availability in a twofold sense: firstly, the commercial availability of a wide spectrum of pure, active, and safe growth factors with the correct folding and posttranslational modifications, and secondly, the *bio*availability at the right dosage with the right timing at the desired site of action. Bonadio et al. have pointed out that, because of the often short half-lives of recombinant proteins, in particular of growth factors, their therapeutic application requires high local dosage with the risk of local and systemic toxicity. The desired local response may fade quickly, while protracted action may be required [114]. Unlike growth factors as proteins, the cDNA sequences of the known growth factors are readily available. Therefore, genetic manipulation

of cells to express the desired factor(s) and their transplantation *per se* [260] or grown on biomaterial scaffolds [261, 262] have emerged as successful alternatives to the local application of growth factor proteins. Matrices loaded with naked plasmid DNA coding for BMP-4 (bone morphogenetic protein 4) and/or parathyroid hormone have successfully been applied in small and large animal models of bone healing [114–116]. Kyriakides et al. [117], Tyrone et al. [118], Berry et al. [119], Pakkanen et al. [120], Chandler et al. [121], Gu et al. [122], and Doukas et al. [123] have used collagen as carrier for plasmid–DNA, polylysine–DNA, lipid–DNA, and adenoviruses in various wound healing and tissue engineering models. These studies clearly demonstrate the feasibility and therapeutic efficacy (in animal models) of the gene-activated matrix concept in tissue engineering.

Although naked DNA transfects *in vivo*, its utility in matrix-mediated gene delivery may be limited if it is rapidly released from the carrier material in an unprotected form. As we know, complexation of DNA with polycations or cationic lipids protects it from degradation and can enhance transfection. On the other hand, such vectors are subject to opsonization *in vivo*. If protracted transfection in the context of the gene-activated matrix concept is desired, then a combination of sustained vector release and stability is probably required. We have previously developed protective copolymers to shield vectors from undesired interactions *in vivo* [77]. These compounds are strictly alternating copolymers of PEG and peptide derivatives. In contrast to other approaches, in which the shielding layer is attached to vector core particles in a covalent manner [78, 175], protective copolymers are attached through electrostatic interaction. One advantage of this concept is its flexibility and versatility, because the attachment of the protective layer requires nothing more than mixing with a preformed vector particle without additional chemical reactions and purification steps (Figure 5.8).

Both the covalent and noncovalent approaches protect vectors from undesired interactions and are compatible with gene delivery. We have examined the utility copolymer-protected gene vectors (COPROGs) in the gene-activated matrix concept, loading collagen sponges by simple incubation followed by freeze-drying with various vectors (naked DNA, PEI–DNA, DOTAP/cholesterol–DNA, COPROGs) and compared their release profiles and transfecting capacities *in vitro* and *in vivo* [113].

Figure 5.8 Copolymer-protected gene vectors (*COPROGs*) are assembled from polycation-compacted DNA particles (here: branched PEI–DNA; box in center of figure) and protective copolymers by electrostatic interaction. The synthetic procedure for protective copolymers is shown here schematically and has been described elsewhere [77]. Briefly, 3-(2′-pyridyldithio)-propionic acid (**1**) is treated with *tert*-butyl-protected glutamic acid under N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide/1-hydroxybenzotriazole activation. The product is deprotected with trifluoroacetic acid to yield compound **2**, which is copolymerized with O,O′-bis(2-aminoethyl)-poly(ethylene glycol) (here: average molecular weight 6000 Da) with dicyclohexylcarbodiimide activation. After purification by size exclusion chromatography, the reactive copolymer backbone is treated with the peptide "YE5C" (sequence $[Ac\text{-}YE_5]_2K$-ahx-C; ahx = 6-aminohexanoic acid). Product **3** thus consists of a PEG backbone (shaded dark gray) and peptide side chains consisting of an anionic moiety (shaded light gray) linked to the backbone through a spacer (shaded intermediate gray). *COPROGs* are used to prepare vector-loaded collagen sponges (compare Figure 5.7) or fibrinogen components.

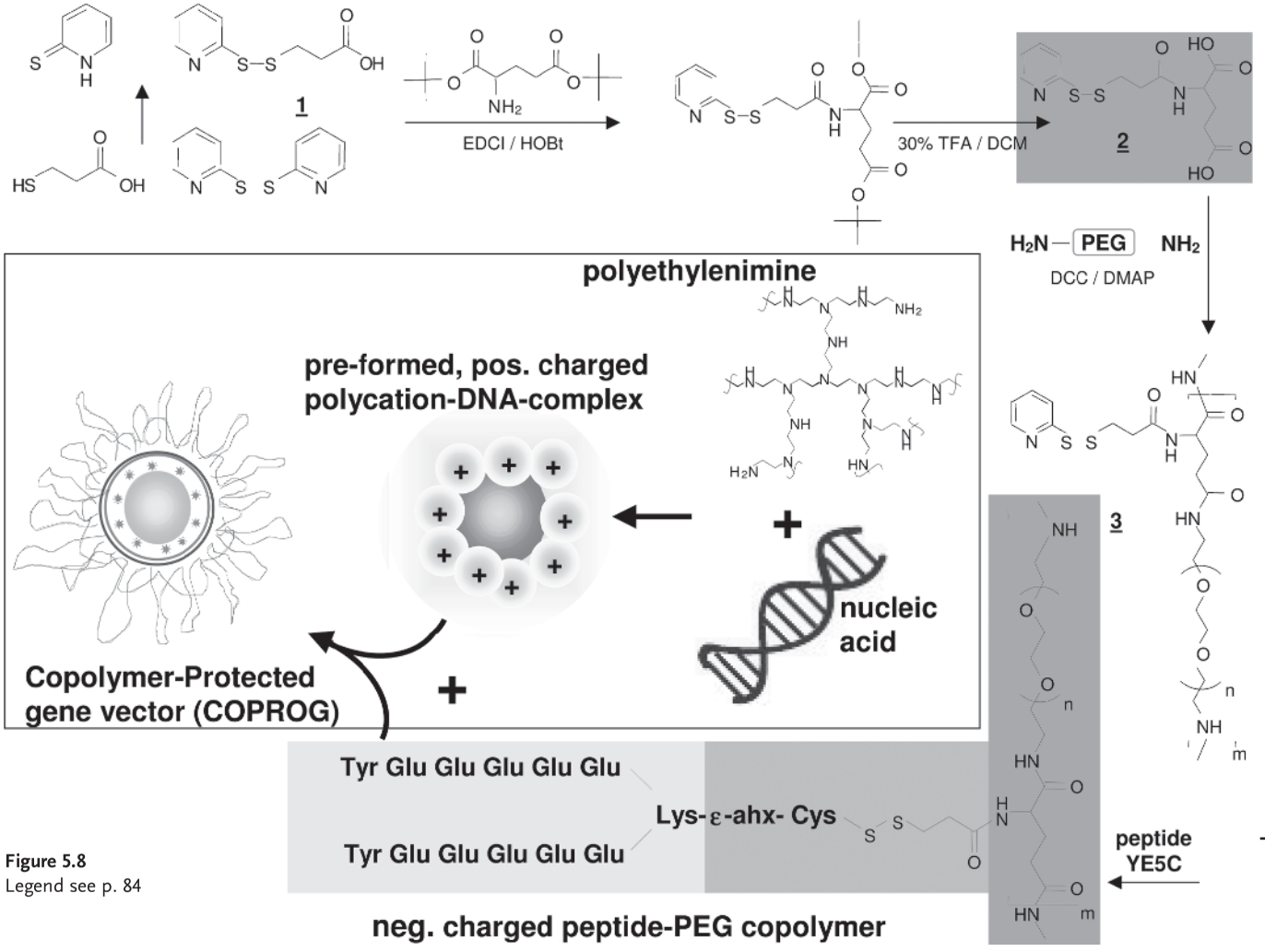


Figure 5.8
Legend see p. 84

Even at a low ratio of DNA to carrier material (about 10 μg DNA per mg collagen) we found that about 77% of the loaded dose was rapidly released in aqueous buffer in an initial burst in the case of naked DNA. In contrast, the same DNA dose in PEI–DNA or DOTAP/cholesterol–DNA formulation remained more tightly associated with the carrier material and was continuously released over several weeks. Probably because of the shielding effect, COPROGs were less tightly associated than unprotected vectors, 27% being released in an initial burst, followed by an exponential release profile over several weeks. These data were then correlated with reporter gene expression mediated by the vector loaded carrier materials. Naked DNA gave rise to low level expression over a short period of time (7 days). The examined DNA formulations yielded substantially higher (several orders of magnitude) and persistent reporter gene expression levels (up to 8 weeks, the maximum duration of the experiments). The highest expression levels were observed with COPROGs. Consistently with the release profiles, these formulations transfected both cells colonizing the sponges and surrounding cells. Upon subcutaneous implantation in rats, only the COPROG-loaded sponges gave rise to reproducible reporter gene expression for at least seven days in cells colonizing the sponges. Naked DNA was completely inactive in this setup [113]. We conclude that, depending on the site of implantation, naked DNA is lost for gene delivery because of the rapid release and degradation of the major part of the loaded dose. DNA in PEI or lipid formulations is protected from degradation and so is superior in short-term transfection, but only vectors that are to some extent resistant to opsonization will be suitable for sustained localized delivery.

In a recent, still unpublished, study we used fibrin instead of collagen as a carrier material and obtained similar results. In this case the carrier was designed as a fibrin glue, which can be applied as an injectable implant. The vector is formulated and freeze-dried together with the fibrinogen component of a commercially available fibrin glue in clinical use as a tissue sealant. Before use, it is treated in exactly the same manner as the parent fibrin glue. The fibrinogen and thrombin components are rehydrated and applied to the target area, such as a skin, bone, or cartilage defect. Optionally, one component of the vector-loaded glue can be premixed with cells (usually autologous) appropriate for colonizing the tissue defect (e.g., keratinocytes for skin wounds or chondrocytes for cartilage defects). In this setup we also observed a rapid release of naked DNA and low and slow release of COPROGs, consistently with little or no transfected gene expression with the former and high and persistent expression with the latter. These results are encouraging in the light of a recent study carried out by Christman et al. [112]. These researchers had previously shown that the injection of a fibrin glue preserved left ventricular geometry and prevented deterioration of cardiac function following myocardial infarction in an animal model. When they formulated the fibrin glue with plasmid DNA coding for pleiothrophin, they observed increased neovasculature formation in the myocardium relative to that seen with direct injection of naked plasmid DNA in saline. These results are consistent with previous observations of the transfection-enhancing effect of fibrin [263]. From our own results we conclude that biomaterial vector composites with well balanced profiles of release and vector

protection display strong potential in localized tissue repair supported by the transfection of growth factor genes.

In the examples discussed above, composites of nucleic acid formulations and biomaterials served as gene-activated implants. An extension of this strategy is to combine nucleic acid formulations and biomaterials with classical medical implants such as stents or metallic bone implants. Isner et al. were already reporting about the therapeutically successful administration of naked DNA coding for vascular endothelial growth factor (VEGF) to an artery of a patient suffering from severe limb ischemia in 1996 [264]. DNA was coated to an angioplasty balloon in combination with a hydrogel polymer. DNA delivery to the artery was accomplished by inflating the balloon, representing a double example of the application of physical force to achieve localized nucleic acid delivery.

Surface-coated drug eluting stents are used with great success in the prevention of restenosis [265, 266]. Similarly, bone implants provided with antibiotic-releasing surface coatings have been developed to prevent implant-associated infections [267, 268]. Surface coatings of metallic implants with growth factors have been used in animal models to promote bone healing [269–272]. The use of nucleic acids as prodrugs also offers great potential in these approaches. There are various ways of coating surfaces with nucleic acids. It has been shown recently that coprecipitation of DNA with inorganic minerals (actually a new modification or extension of the old calcium phosphate precipitation method) is a useful method by which to prepare transfection-active surfaces [273]. In other approaches, nucleic acids or vectors are dispersed in solutions of polymers coated with implant materials [110]. Examples are vector or nucleic acid emulsions in polyurethane [124] or polylactide or polylactide-co-glycolide organic solutions [110, 125]. phVEGF 2-plasmid-coated "BiodivYsio phosphorylcholine polymer" stents have been demonstrated to be a powerful alternative to drug-eluting stents for restenosis inhibition [274].

5.5.8
Injectable Implants for Localized Nucleic Acid Delivery

Whereas controlled release formulations such as those described above are first formed *ex vivo* and are then inserted into the body, a novel approach focuses on the formation of the biodegradable implant *in situ* upon injection. Such controlled delivery formulations have been termed injectable polymeric implants. Biomaterials such as fibrin glue [112] or collagen solutions, as described above, can be used for this purpose. In another approach, an injectable polymeric implant is made up of a water-insoluble biodegradable polymer dissolved in a pharmaceutically acceptable water-miscible solvent and the biologically active drug. Upon injection of the polymeric solution, the water-miscible solvent diffuses away in the surrounding tissue and the polymer begins to precipitate, forming the solid implant matrix. As the implant matrix solidifies, the biologically active ingredient is encapsulated within the polymer matrix. The result of this process is a defined polymer matrix containing the desired biologically active drug encapsulated in an implant formed *in situ* in the body. The release mechanisms from the injectable polymeric implants are similar

to the solid polymer-based controlled release systems formed *ex vivo*. Such an injectable polymeric implant, consisting of poly(lactide-co-glycolid) (PLGA) and glycofurol as solvent, was successfully used to encapsulate various plasmid DNAs [111]. *In vivo* injection of polymer solutions variously containing a plasmid coding for the luciferase gene, secreted human placental alkaline phosphatase, or developmental endothelial locus, into the subcutaneous flank tissue of mice resulted in successful in situ formation of implants and robust gene expression from surrounding cells. Sustained gene expression for more than 60 days after implantation was observed only when plasmid DNA was formulated as an injectable implant, but not upon injection of an aqueous plasmid DNA solution. In addition, a visible increase in blood vessel formation containing erythrocytes could be demonstrated around the injection site of injectable implants containing the developmental endothelial locus gene [111].

Another type of injectable implants – microparticles comprising vectors or DNA, mostly used for vaccination purposes – has already been mentioned. The most strongly established in this respect are microparticulates of hydrolytically degradable polyesters such as PLGA. DNA can be associated with such particulates by various methods, including emulsification processes or adsorption onto preformed particulates [275]. The coformulation protects DNA from degradation and also serves as a controlled release composition. Microspheres (1–10 μm) provide an opportunity to target phagocytotic cells preferentially, due to selective uptake by such cells, so these formulations can be used for the selective transfection of antigen-presenting cells (APCs) such as dendritic cells. Upon expression of the transfected antigen gene, processed fragments are displayed by MHC class I and class II complexes. Depending on the formulation of the microparticulates and the mode of administration, potent immune responses of various types can be elicited and, interestingly, immune tolerance can also be induced with appropriate compositions [276]; the reader is referred to a recent review volume for details [194]. Wang et al. have recently reported the use of poly(ortho ester) microspheres for DNA vaccination purposes. According to these authors, these compounds are superior to PLGA microspheres in that they display surface-confined erosion in response to acidic pH (in contrast to bulk erosion in the case of PLGA), release intact DNA in a timed manner, and do not produce aggressive (acidic) hydrolysis products [277].

The ballistic methods of nucleic acid delivery reviewed elsewhere (Chapter 10) [91, 278, 279] are comparable to the administration of vector-loaded microspheres, whilst the gene gun approach is also extensively used for vaccination studies. This approach reportedly generates better results than other delivery methods to the skin [280], but it is probably too early to judge which of the many approaches to localized gene and drug delivery is best for a given application.

5.5.9
Aerosol Application of Nucleic Acids

The lung represents an attractive organ for application of therapeutic gene delivery vectors to treat various inherited or acquired pulmonary diseases such as cystic fibrosis, α_1-antitrypsin deficiency, asthma, or lung cancer (see also Chapter 8).

In principle, the lung can be targeted from two different sites of application: either the vasculature, through intravenous application, or topically, from the luminal side (i.e., the organ surface exposed to the environmental air). There are various reasons why topical gene vector application from the luminal side seems more attractive than intravenous application and thus represents the method of choice for localized nucleic acid delivery to the lungs.

1. Unlike intravenous application, topical gene vector application is noninvasive; inhalation is well accepted by patients.
2. Unlike topical gene vector application, intravenous application favors gene expression in the alveolar epithelium of the lung, thereby targeting the alveolar epithelial type II cells but not the bronchial epithelium [281]. Moreover, i.v. administered vectors are not selective, in that they also transfect endothelial cells of the vasculature of the lung parenchyma, and also other nontarget organs such as kidneys, spleen, liver, and heart [171, 282–284].

In principle, alveolar type II cells represent an important target for gene therapy due to their progenitor cell character and their proliferative potential. These cells are a reservoir of regenerative stem cells of the alveolar lung tissue [285]. A single integrational transfection in this cell type should be sufficient to restore functionality of the transfected cell for the remaining lifespan of the treated individual. However, other local regions of the lung, such as the bronchial epithelium, might be an even more important target. The bronchial epithelium plays an important role in various widespread diseases, such as lung cancer or cystic fibrosis, and can hardly be targeted by systemic gene vector application. Intravenous vector application cannot be considered as a suitable method to target lung tissue selectively. In particular, the lack of loco-regional control of gene vector targeting to the lung upon intravenous application raises safety concerns, as evidenced by systemic side effects discussed below.

As discussed in detail earlier in this chapter, the goal of drug targeting is to deliver the minimum necessary quantities of pharmaceutically active drugs selectively to the diseased site of an affected individual in order to induce the desired therapeutic effect and at the same time to minimize potentially hazardous side effects at nondiseased sites. With respect to drug delivery to the lungs, the ultimate goal should be to deliver drugs to either the alveolar or bronchial epithelium at therapeutic doses whilst avoiding systemic toxic drug concentrations. Studies in which the benefits of topical drug application to the lungs, as compared with systemic drug application, were demonstrated to achieve this goal have very recently been published. Levels of cytotoxic ^{14}C-tagged doxorubicin in the lungs of dogs were more than one order of magnitude higher when equal doses were administered topically by aerosol application than after intravenous application. In addition, radioactivity levels in the lungs remained high for several days, whilst systemic levels of radioactivity were low in relation to intravenous application, demonstrating the superior properties of aerosol application over systemic application for targeting the lung [286]. In particular, the low systemic levels of doxorubicin should result in

reduced systemic toxicity and side effects. These observations were further supported by a mouse study in which paclitaxel was formulated with liposomes and equal doses were administered either intravenously or topically by aerosol to the lungs [287]. The levels of drug measured in the lungs of mice after aerosol treatment were 26 times higher than those observed after systemic application. Such preferential lung-targeted drug delivery has not only been observed for small drugs but also for plasmid DNA formulated with nonviral gene carriers. Both intratracheal instillation and aerosol delivery of PEI-based gene vectors to the lungs of mice resulted in gene expression restricted to the lungs [107, 288, 289]. It is important to note that the inflammatory response to PEI–DNA gene vectors after aerosol delivery was significantly lower than that observed after intravenous application at equal doses and was only restricted to the lung, as evidenced by a lack of any increase in cytokine levels in the serum [290]. High toxicity of PEI–DNA gene vectors has been observed at the high gene vector doses necessary for efficient gene expression in the lungs after systemic application, as evidenced by high mortality rates [79]. Analysis of the biodistribution patterns and pharmacokinetics of PEI–DNA complexes either applied systemically or by aerosol application demonstrated a lung-specific area under the curve 2.8 times larger for gene vectors aerosolized to the lung than for systemically applied gene vectors. In addition, and in contrast with systemic application, other organs did not show amounts of intact plasmid DNA distinguishable from those in untreated mice after aerosol application as examined by RT-PCR [106]. Only nanogram quantities of plasmid DNA delivered to the lungs of mice were needed to transfect the airway epithelium of large airways efficiently. Interestingly, aerosol application to mice lungs was three orders of magnitude more efficient than direct intratracheal instillation when standardized to the dose of plasmid DNA delivered to the lungs [291].

Taken together, these data demonstrate that aerosol application represents the method of choice for localized nucleic acid delivery to the lungs. This is particularly in evidence in the superior dose–response relationship and toxicity profile of localized gene delivery to the lungs upon aerosol application, relative to systemic application.

5.5.10
Use of Ultrasound to Trigger Localized Delivery

Biological control of drug action can be exploited if, for example, the structure or a biological process a drug is designed to act on is prevalent or overexpressed only or predominantly in a tissue of choice or if a biological process required for drug action can be induced locally in a target tissue. Additionally, drugs can be designed as prodrugs designed to be converted into the active drug only or predominantly in a target tissue. In many, if not most, approaches in gene therapy, the administered nucleic acid, no matter whether it is in the context of a viral or a nonviral vector or is in “naked” form, can be regarded as a prodrug because the actual therapeutic agent is the product of its expression. Gene therapeutics can be made tissue-specific by virtue of tissue-specific sequences such as tissue-specific promoters or other

control elements (transcriptional targeting). Drugs can be formulated in a manner that requires some activation step either to release the active drug and/or to transform its prodrug configuration into the active form and/or to induce a desired interaction with the target tissue.

Physical principles can be used to perform such activation steps and so control drug action locally. Such principles include the application of electric fields, magnetic fields, radiation (electromagnetic and particulate), and acoustic waves (ultrasound). Some examples are electroporation for nucleic acid delivery, local induction of hyperthermia to mediate drug release from temperature-sensitive formulations by use of microwaves or, for example, local application of alternating magnetic fields with local injection of magnetic particles [292, 293], exploitation of radiation-sensitive promoters to control the expression of transfected genes [135], or local application of ultrasound in order to induce drug release from liposomes or microbubbles [139].

Microbubbles are gas-filled microspheres originally developed as contrast agents for medical ultrasound imaging purposes. Micrometer-sized gas bubbles that resonate at a diagnostic frequency are ideal reflectors for ultrasound [294]. For application in systemic circulation, these bubbles should be smaller than 5–7 μm, in order not to obstruct blood capillaries. In the simplest case, microbubbles are nothing more than air bubbles [295, 296] or gas emulsions [297] in an aqueous phase. Such bubbles can be stabilized if the air–liquid interface is provided with a shell. The shell may consist of renografin, indocyanin green, carbohydrates such as dextrose, proteins, denatured proteins, surfactants, lipids, or synthetic polymers such as polylactides [298, 299]. Various compositions are discussed in comprehensive reviews [137, 139]. First-generation microbubbles, which were air-filled, suffered from limited stability. Upon intravenous infusion, the air dissolves rapidly in the blood, so the bubbles are lost for imaging or drug delivery. The physical background for these phenomena has been discussed by Schutt et al. [294]. Use of gases with low Ostwald coefficients greatly improves bubble stability, and perfluorocarbons have turned out to be ideal gases for microbubble preparation, thanks to their low aqueous solubilities and sufficient volatilities. More recent compositions are so-called nanoemulsions consisting of a bubble shell filled with a liquid perfluorocarbon. These compositions can be designed in such a manner that the fluorocarbon will undergo a phase transition from liquid to gaseous states at a range of different temperatures [139]. EchoGen (Sonus Pharmaceuticals Inc., USA) was a composition of an emulsion of perfluorinated *n*-pentane in water, this perfluorocarbon converting into a gas at body temperature (boiling point 29 °C). Preparation procedures for microbubbles include simple shaking, emulsion procedures, application of shear forces, or sonication, and can be found in the scientific and patent literature.

Like gene vectors and liposomes, microbubbles can be targeted by exploiting receptor–ligand-type interactions (including antigen–antibody interactions) [137, 139]. In this manner, functional molecular imaging can be carried out with the aid of ultrasound.

Microbubbles have been used as drug carriers. Both low molecular weight drugs and high molecular weight drugs such as nucleic acids can be associated with

microbubbles. The association can be achieved in various ways: the drug can be bound covalently or noncovalently to the surface of a bubble shell, it can be integral part of the shell itself, or it can reside in the interior of the bubble. In all cases, drug molecules are able to interact through chemical bonds or physically (noncovalently) with each other and/or with other components of the shell. For association with microbubbles, a drug may also be provided in the form of a prodrug [300, 301]. The various modes of drug association have been discussed in comprehensive reviews [137, 139, 302, 303].

Drug-loaded microbubbles offer potential as "magic bullet" agents with which to deliver drugs to precise locations in the body, these precise locations being determined by where the ultrasound energy is focused [139]. The physical basis is that gas-filled microbubbles can be induced to "pop" by use of ultrasound of appropriate frequency and energy. Ultrasound probes operating in the low MHz range have been found to be optimal for this purpose [304]. The interaction of microbubbles and ultrasound results in cavitation, bubble burst, and consequent drug release. In addition, cavitation can result in microvessel rupture and hence increased permeability of the endothelial barrier [305]. This effect has been used to deliver nanoparticles and red blood cells to the interstitium of rat skeletal muscle [306]. Cavitation nuclei formed by microbubbles have also been used to permeabilize the blood–brain barrier [307]. It can be envisaged that simple codelivery of a drug with microbubbles and local ultrasound irradiation may be sufficient to achieve locally enhanced delivery (i.e., the drug to be delivered may not need to be associated with microbubbles) [139].

Microbubbles have been used successfully in nucleic acid delivery [137, 139, 140]. Interestingly, ultrasound alone has been shown to enhance gene delivery to cell lines [308, 309], to skeletal muscle [310], and to tumors [311]. Associating nucleic acids with microbubbles and applying such compositions *in vitro* and *in vivo* with exposure of the target tissue to ultrasound is a highly effective method for triggering localized delivery of nucleic acids and drugs in general in a variety of tissues [138, 301, 312–321].

Microbubbles appear to be one of the most promising examples in which biological and physical principles of targeting and control can be combined in a manner such that drug delivery can also be remote-controlled by an external physical force.

5.6 Concluding Remarks

The initial concepts of gene therapy were conceived almost forty years ago [3]. The validity and therapeutic efficacy of the concept have been demonstrated in humans with viral vectors [322], but this success has been overshadowed by severe adverse events [323]. This and another tragic setback to nucleic acid therapy concepts, the death of a treated patient [324], highlight how little we understand the complex biology constituting the basis of the therapeutic concept we pursue. Despite more

than 30 years of continued efforts, the final breakthrough in nucleic acid-based therapies has yet to be achieved. These decades of research have greatly contributed, however, to improved understanding of the biology involved and to an appreciation of the complex challenges presented by nucleic acid delivery. Efficient synthetic alternatives to viruses as nucleic acid shuttles are available and nucleic acid delivery has become an important research tool in the biomedical sciences. With regard to the important major step still to be taken towards efficient and widely applicable nucleic acid-based therapies, we now know that it will have to be taken by an interdisciplinary effort. Medical, pharmaceutical, chemical, biological, and – importantly – also physical aspects will need to be considered and the associated scientific efforts will need to be united in order to generate safe and efficient nucleic acid pharmaceuticals. The ability to localize delivery is an important step in this direction, in terms both of efficacy and of safety. It is likely that a combination of physical control of delivery, of localization, and of activation with the corresponding biological concepts will be the way to success.

References

1 Mello, C. C., Conte, D., Jr., Revealing the world of RNA interference. *Nature* **2004**, *431(7006)*, 338–342.
2 He, L., Hannon, G. J., MicroRNAs: small RNAs with a big role in gene regulation. *Nat. Rev. Genet.* **2004**, *5(7)*, 522–531.
3 Tatum, E. L., Molecular biology, nucleic acids, and the future of medicine. *Perspect. Biol. Med.* **1966**, *10(1)*, 19–32.
4 Jackson, D. A., Symons, R. H., Berg, P., Biochemical method for inserting new genetic information into DNA of Simian Virus 40: circular SV40 DNA molecules containing lambda phage genes and the galactose operon of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **1972**, *69(10)*, 2904–2909.
5 Wolff, J. A., Malone, R. W., Williams, P., Chong, W., Acsadi, G., Jani, A., Felgner, P. L., Direct Gene Transfer Into Mouse Muscle Invivo. *Science* **1990**, *247(4949)*, 1465–1468.
6 Tovell, D. R., Colter, J. S., Observations on the assay of infectious viral ribonucleic acid: effects of DMSO and DEAE-dextran. *Virology* **1967**, *32(1)*, 84–92.
7 Graham, F. L., Van der Eb, A. J., Transformation of rat cells by DNA of human adenovirus 5. *Virology* **1973**, *54(2)*, 536–539.
8 Nicolau, C., Cudd, A., Liposomes As Carriers Of DNA. *Critical Reviews in Therapeutic Drug Carrier Systems* **1989**, *6(3)*, 239–271.
9 Sober, H. A., Schlossman, S. F., Yaron, A., Latt, S. R. G., Protein-nucleic acid interaction. I. Nuclease-resistant polylysine-ribonculeic acid complexes. *Biochemistry* **1966**, *5(11)*, 3608–3616.
10 Latt, S. A., Sober, H. A., Protein-nucleic acid interactions. 3. Cation effect on binding strength and specificity. *Biochemistry* **1967**, *6(10)*, 3307–3314.
11 Latt, S. A., Sober, H. A., Protein-nucleic acid interactions. II. Oligopeptide-polyribonucleotide binding studies. *Biochemistry* **1967**, *6(10)*, 3293–3306.

12 Latt, S. A., Sober, H. A., Protein-nucleic acid interactions. III. Cation effect on binding strength and specificity. *Biochemistry* **1967**, *6(10)*, 3307–3314.
13 Porschke, D., The binding of Arg- and Lys-peptides to single stranded polyribonucleotides and its effect on the polymer conformation. *Biophysical Chemistry* **1979**, *10(1)*, 1–16.
14 Haynes, M., Garrett, R. A., Gratzer, W. B., Structure of Nucleic Acid-Polybase Complexes. *Biochemistry* **1970**, *9*, 4410–4416.
15 Gosule, L. C., Schellman, J. A., Compact form of DNA induced by spermidine. *Nature* **1976**, *259(5541)*, 333–335.
16 Gosule, L. C., Schellman, J. A., DNA condensation with polyamines. I. Spectroscopic studies. *Journal of Molecular Biology* **1978**, *121(3)*, 311–326.
17 Wu, G. Y., Wu, C. H., Receptor-mediated *in vitro* gene transformation by a soluble DNA carrier system. *J. Biol. Chem.* **1987**, *262*, 4429–4432.
18 Wu, G. Y., Wu, C. H., Receptor-mediated gene delivery and expression *in vivo*. *J. Biol. Chem.* **1988**, *263*, 14621–14624.
19 Wagner, E., Zenke, M., Cotten, M., Beug, H., Birnstiel, M. L., Transferrin-Polycation Conjugates As Carriers For DNA Uptake Into Cells. *Proc. Natl. Acad. Sci. USA* **1990**, *87(9)*, 3410–3414.
20 Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M., Danielsen, M., Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. USA* **1987**, *84(21)*, 7413–7417.
21 Behr, J. P., Demeneix, B. A., Loeffler, J. P., Perez-Mutul, J., Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA. *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 6982–6986.
22 Tang, M. X., Szoka, F. C., The influence of polymer structure on the interactions of cationic polymers with DNA and morphology of the resulting complexes. *Gene Ther.* **1997**, *4(8)*, 823–832.
23 Tang, M. X., Szoka, F. C., Jr. Characterization of polycation complexes with DNA. In: Kabanov, A. V., Felgner, P. L., Seymour, L. (Eds.), Self-Assembling Complexes for Gene Delivery: from Laboratory to Clinical Trial. New York: Wiley and Sons **1998**.
24 Tang, M. X., Li, W., Szoka, F. C., Jr. Toroid formation in charge neutralized flexible or semi-flexible biopolymers: potential pathway for assembly of DNA carriers. *J. Gene Med.* **2005**, *7(3)*, 334–342.
25 Wu, G. Y., Wu, C. H., Evidence for targeted gene delivery to HepG2 hepatoma cells *in vitro*. *Biochemistry* **1988**, *27*, 887–892.
26 Zenke, M., Steinlein, P., Wagner, E., Cotten, M., Beug, H., Birnstiel, M. L., Receptor-Mediated Endocytosis Of Transferrin Polycation Conjugates – An Efficient Way To Introduce DNA Into Hematopoietic Cells. *Proc. Natl. Acad. Sci. USA* **1990**, *87(10)*, 3655–3659.
27 Cotten, M., Langlerouault, F., Kirlappos, H., Wagner, E., Mechtler, K., Zenke, M., Beug, H., Birnstiel, M. L., Transferrin Polycation-Mediated Introduction Of DNA Into Human Leukemic Cells – Stimulation By Agents

That Affect The Survival Of Transfected DNA Or Modulate Transferrin Receptor Levels. *Proc. Natl. Acad. Sci. USA* **1990**, *87(11)*, 4033–4037.

28 Gust, T. C., Zenke, M., Receptor-mediated gene delivery. *ScientificWorldJournal* **2002**, *2*, 224–229.

29 Pardridge, W. M., Brain drug targeting and gene technologies. *Jpn. J. Pharmacol.* **2001**, *87(2)*, 97–103.

30 Varga, C. M., Wickham, T. J., Lauffenburger, D. A., Receptor-mediated targeting of gene delivery vectors: insights from molecular mechanisms for improved vehicle design. *Biotechnol. Bioeng.* **2000**, *70(6)*, 593–605.

31 Wagner, E., Plank, C., Zatloukal, K., Cotten, M., Birnstiel, M. L., Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine–DNA complexes: toward a synthetic virus-like gene-transfer vehicle. *Proc. Natl. Acad. Sci. USA* **1992**, *89(17)*, 7934–7938.

32 Plank, C., Oberhauser, B., Mechtler, K., Koch, C., Wagner, E., The Influence Of Endosome-Disruptive Peptides On Gene Transfer Using Synthetic Virus-Like Gene Transfer Systems. *J. Biol. Chem.* **1994**, *269(17)*, 12918–12924.

33 Plank, C., Zatloukal, K., Cotten, M., Mechtler, K., Wagner, E., Gene Transfer Into Hepatocytes Using Asialoglycoprotein Receptor Mediated Endocytosis Of DNA Complexed With An Artificial Tetra-Antennary Galactose Ligand. *Bioconj. Chem.* **1992**, *3(6)*, 533–539.

34 Ogris, M., Carlisle, R. C., Bettinger, T., Seymour, L. W., Melittin enables efficient vesicular escape and enhanced nuclear access of nonviral gene delivery vectors. *J. Biol. Chem.* **2001**, *276(50)*, 47550–47555.

35 Curiel, D. T., Agarwal, S., Wagner, E., Cotton, M., Adenovirus Enhancement Of Transferrin Polylysine-Mediated Gene Delivery. *Proc. Natl. Acad. Sci. USA* **1991**, *88(19)*, 8850–8854.

36 Wagner, E., Zatloukal, K., Cotten, M., Kirlappos, H., Mechtler, K., Curiel, D. T., Birnstiel, M. L., Coupling Of Adenovirus To Transferrin Polylysine DNA Complexes Greatly Enhances Receptor-Mediated Gene Delivery And Expression Of Transfected Genes. *Proc. Natl. Acad. Sci. USA* **1992**, *89(13)*, 6099–6103.

37 Curiel, D. T., Wagner, E., Cotten, M., Birnstiel, M. L., Agarwal, S., Li, C. M., Loechel, S., Hu, P. C., High-Efficiency Gene Transfer Mediated By Adenovirus Coupled To DNA-Polylysine Complexes. *Human Gene Therapy* **1992**, *3(2)*, 147–154.

38 Cotten, M., Saltik, M., Kursa, M., Wagner, E., Maass, G., Birnstiel, M. L., Psoralen Treatment Of Adenovirus Particles Eliminates Virus Replication And Transcription While Maintaining The Endosomolytic Activity Of The Virus Capsid. *Virology* **1994**, *205(1)*, 254–261.

39 Murthy, N., Robichaud, J. R., Tirrell, D. A., Stayton, P. S., Hoffman, A. S., The design and synthesis of polymers for eukaryotic membrane disruption. *J. Control Release* **1999**, *61(1–2)*, 137–143.

40 Kusonwiriyawong, C., van de Wetering, P., Hubbell, J. A., Merkle, H. P., Walter, E., Evaluation of pH-dependent membrane-disruptive

properties of poly(acrylic acid) derived polymers. *Eur. J. Pharm. Biopharm.* **2003**, *56(2)*, 237–246.

41 Stayton, P. S., Hoffman, A. S., Murthy, N., Lackey, C., Cheung, C., Tan, P., Klumb, L. A., Chilkoti, A., Wilbur, F. S., Press, O. W., Molecular engineering of proteins and polymers for targeting and intracellular delivery of therapeutics. *J. Control Release* **2000**, *65(1–2)*, 203–220.

42 Jones, R. A., Cheung, C. Y., Black, F. E., Zia, J. K., Stayton, P. S., Hoffman, A. S., Wilson, M. R., Poly(2-alkylacrylic acid) polymers deliver molecules to the cytosol by pH-sensitive disruption of endosomal vesicles. *Biochem. J.* **2003**, 372(Pt 1), 65–75.

43 Kyriakides, T. R., Cheung, C. Y., Murthy, N., Bornstein, P., Stayton, P. S., Hoffman, A. S., pH-sensitive polymers that enhance intracellular drug delivery *in vivo*. *J. Control Release* **2002**, *78(1–3)*, 295–303.

44 Cheung, C. Y., Murthy, N., Stayton, P. S., Hoffman, A. S., A pH-sensitive polymer that enhances cationic lipid-mediated gene transfer. *Bioconjug. Chem.* **2001**, *12(6)*, 906–910.

45 Boussif, O., Lezoualc'h, F., Zanta, M. A., Mergny, M. D., Scherman, D., Demeneix, B., Behr, J. P., A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethylenimine. *Proc. Natl. Acad. Sci. USA* **1995**, *92(16)*, 7297–7301.

46 Sonawane, N. D., Szoka, F. C., Jr., Verkman, A. S., Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine–DNA polyplexes. *J. Biol. Chem.* **2003**, *278(45)*, 44826–44831.

47 Haensler, J., Szoka, F. C., Polyamidoamine Cascade Polymers Mediate Efficient Transfection Of Cells in Culture. *Bioconj. Chem.* **1993**, *4(5)*, 372–379.

48 Tang, M. X., Redemann, C. T., Szoka, F. C., *In vitro* Gene Delivery by Degraded Polyamidoamine Dendrimers. *Bioconj. Chem.* **1996**, *7(6)*, 703–714.

49 Boussif, O., Delair, T., Brua, C., Veron, L., Pavirani, A., Kolbe, H. V., Synthesis of polyallylamine derivatives and their use as gene transfer vectors *in vitro*. *Bioconjug. Chem.* **1999**, *10(5)*, 877–883.

50 Van Craynest, N., Santaella, C., Boussif, O., Vierling, P., Polycationic telomers and cotelomers for gene transfer: synthesis and evaluation of their an vitro transfection efficiency. *Bioconjug. Chem.* **2002**, *13(1)*, 59–75.

51 Midoux, P., Monsigny, M., Efficient gene transfer by histidylated polylysine pDNA complexes. *Bioconj. Chem.* **1999**, *10(3)*, 406–411.

52 Pack, D. W., Putnam, D., Langer, R., Design of imidazole-containing endosomolytic biopolymers for gene delivery. *Biotechnol. Bioeng.* **2000**, *67(2)*, 217–223.

53 Putnam, D., Gentry, C. A., Pack, D. W., Langer, R., Polymer-based gene delivery with low cytotoxicity by a unique balance of side-chain termini. *Proc. Natl. Acad. Sci. USA* **2001**, *98(3)*, 1200–1205.

54 Lim, Y. B., Kim, S. M., Suh, H., Park, J. S., Biodegradable, endosome disruptive, and cationic network-type polymer as a highly efficient and nontoxic gene delivery carrier. *Bioconjug. Chem.* **2002**, *13(5)*, 952–957.

55 Lim, D. W., Yeom, Y. I., Park, T. G., Poly(DMAEMA-NVP)-b-PEG-galactose as gene delivery vector for hepatocytes. *Bioconjug. Chem.* **2000**, *11(5)*, 688–695.

56 Kichler, A., Sabourault, N., Decor, R., Leborgne, C., Schmutz, M., Valleix, A., Danos, O., Wagner, A., Mioskowski, C., Preparation and evaluation of a new class of gene transfer reagents: poly(-alkylamino-siloxanes). *J. Control Release* **2003**, *93(3)*, 403–414.

57 Coeytaux, E., Coulaud, D., Le Cam, E., Danos, O., Kichler, A., The cationic amphipathic alpha-helix of HIV-1 viral protein R (Vpr) binds to nucleic acids, permeabilizes membranes, and efficiently transfects cells. *J. Biol. Chem.* **2003**, *278(20)*, 18110–18116.

58 Kichler, A., Leborgne, C., Marz, J., Danos, O., Bechinger, B., Histidine-rich amphipathic peptide antibiotics promote efficient delivery of DNA into mammalian cells. *Proc. Natl. Acad. Sci. USA* **2003**, *100(4)*, 1564–1568.

59 van de Wetering, P., Cherng, J. Y., Talsma, H., Crommelin, D. J., Hennink, W. E., 2-(Dimethylamino)ethyl methacrylate based (co)polymers as gene transfer agents. *J. Control Release* **1998**, *53(1–3)*, 145–153.

60 Luten, J., van Steenis, J. H., van Someren, R., Kemmink, J., Schuurmans-Nieuwenbroek, N. M., Koning, G. A., Crommelin, D. J., van Nostrum, C. F., Hennink, W. E., Water-soluble biodegradable cationic poly-phosphazenes for gene delivery. *J. Control Release* **2003**, *89(3)*, 483–497.

61 Xu, Y., Szoka, F. C., Jr. Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. *Biochemistry* **1996**, *35(18)*, 5616–5623.

62 Zelphati, O., Szoka, F. C., Jr. Mechanism of oligonucleotide release from cationic liposomes. *Proc. Natl. Acad. Sci. USA* **1996**, *93(21)*, 11493–11498.

63 Zelphati, O., Szoka, F. C., Jr. Intracellular distribution and mechanism of delivery of oligonucleotides mediated by cationic lipids. *Pharm. Res.* **1996**, *13(9)*, 1367–1372.

64 Pedroso de Lima, M. C., Simoes, S., Pires, P., Faneca, H., Duzgunes, N., Cationic lipid–DNA complexes in gene delivery: from biophysics to biological applications. *Adv. Drug Deliv. Rev.* **2001**, *47(2–3)*, 277–294.

65 Lin, A. J., Slack, N. L., Ahmad, A., George, C. X., Samuel, C. E., Safinya, C. R., Three-dimensional imaging of lipid gene-carriers: membrane charge density controls universal transfection behavior in lamellar cationic liposome–DNA complexes. *Biophys. J.* **2003**, *84(5)*, 3307–3316.

66 Ewert, K., Slack, N. L., Ahmad, A., Evans, H. M., Lin, A. J., Samuel, C. E., Safinya, C. R., Cationic lipid–DNA complexes for gene therapy: understanding the relationship between complex structure and gene delivery pathways at the molecular level. *Curr. Med. Chem.* **2004**, *11(2)*, 133–149.

67 Stoll, S. M., Calos, M. P., Extrachromosomal plasmid vectors for gene therapy. *Curr. Opin. Mol. Ther.* **2002**, *4(4)*, 299–305.

68 Conese, M., Auriche, C., Ascenzioni, F., Gene therapy progress and prospects: episomally maintained self-replicating systems. *Gene Ther.* **2004**, *11(24)*, 1735–1741.

69 Recchia, A., Perani, L., Sartori, D., Olgiati, C., Mavilio, F., Site-specific integration of functional transgenes into the human genome by adeno/AAV hybrid vectors. *Mol. Ther.* **2004**, *10(4)*, 660–670.

70 Groth, A. C., Olivares, E. C., Thyagarajan, B., Calos, M. P., A phage integrase directs efficient site-specific integration in human cells. *Proc. Natl. Acad. Sci. USA* **2000**, *97(11)*, 5995–6000.

71 Thyagarajan, B., Olivares, E. C., Hollis, R. P., Ginsburg, D. S., Calos, M. P., Site-specific genomic integration in mammalian cells mediated by phage phi C31 integrase. *Molecular and Cellular Biology* **2001**, *21(12)*, 3926–3934.

72 Plank, C., Mechtler, K., Szoka, F. C., Wagner, E., Activation of the Complement System by Synthetic DNA Complexes: A Potential Barrier for Intravenous Gene Delivery. *Hum. Gene Ther.* **1996**, *7(12)*, 1437–1446.

73 Krieg, A. M., CpG motifs in bacterial DNA and their immune effects. *Annu. Rev. Immunol.* **2002**, *20*, 709–760.

74 Williams, B. R., Role of the double-stranded RNA-activated protein kinase (PKR) in cell regulation. *Biochem. Soc. Trans.* **1997**, *25(2)*, 509–513.

75 Kursa, M., Walker, G. F., Roessler, V., Ogris, M., Roedl, W., Kircheis, R., Wagner, E., Novel shielded transferrin-polyethylene glycol-polyethylenimine/DNA complexes for systemic tumor-targeted gene transfer. *Bioconjug. Chem.* **2003**, *14(1)*, 222–231.

76 Ogris, M., Walker, G., Blessing, T., Kircheis, R., Wolschek, M., Wagner, E., Tumor-targeted gene therapy: strategies for the preparation of ligand-polyethylene glycol-polyethylenimine/DNA complexes. *J. Control Release* **2003**, *91(1–2)*, 173–181.

77 Finsinger, D., Remy, J. S., Erbacher, P., Koch, C., Plank, C., Protective copolymers for nonviral gene vectors: synthesis, vector characterization and application in gene delivery. *Gene Ther.* **2000**, *7(14)*, 1183–1192.

78 Oupicky, D., Ogris, M., Howard, K. A., Dash, P. R., Ulbrich, K., Seymour, L. W., Importance of lateral and steric stabilization of polyelectrolyte gene delivery vectors for extended systemic circulation. *Mol. Ther.* **2002**, *5(4)*, 463–472.

79 Chollet, P., Favrot, M. C., Hurbin, A., Coll, J. L., Side-effects of a systemic injection of linear polyethylenimine–DNA complexes. *J. Gene Med.* **2002**, *4(1)*, 84–91.

80 Plank, C., Anton, M., Rudolph, C., Rosenecker, J., Krotz, F., Enhancing and targeting nucleic acid delivery by magnetic force. *Expert Opin. Biol. Ther.* **2003**, *3(5)*, 745–758.

81 Zabner, J., Fasbender, A. J., Moninger, T., Poellinger, K. A., Welsh, M. J., Cellular And Molecular Barriers To Gene Transfer By A Cationic Lipid. *J. Biol. Chem.* **1995**, *270(32)*, 18997–19007.

82 Kichler, A., Leborgne, C., Coeytaux, E., Danos, O., Polyethylenimine-mediated gene delivery: a mechanistic study. *J. Gene Med.* **2001**, *3(2)*, 135–144.

83 Pollard, H., Remy, J. S., Loussouarn, G., Demolombe, S., Behr, J. P., Escande, D., Polyethylenimine but not cationic lipids promotes transgene

delivery to the nucleus in mammalian cells. *J. Biol. Chem.* **1998**, *273(13)*, 7507–7511.

84 Lubbe, A. S., Alexiou, C., Bergemann, C., Clinical applications of magnetic drug targeting. *J. Surg. Res.* **2001**, *95(2)*, 200–206.

85 Wagner, E., Kircheis, R., Walker, G. F., Targeted nucleic acid delivery into tumors: new avenues for cancer therapy. *Biomed. Pharmacother.* **2004**, *58(3)*, 152–161.

86 Takakura, Y., Nishikawa, M., Yamashita, F., Hashida, M., Influence of physicochemical properties on pharmacokinetics of non-viral vectors for gene delivery. *J. Drug. Target.* **2002**, *10(2)*, 99–104.

87 Boulikas, T., Nuclear localization signal peptides for the import of plasmid DNA in gene therapy (review). *International Journal Of Oncology* **1997**, *10(2)*, 301–309.

88 Cartier, R., Reszka, R., Utilization of synthetic peptides containing nuclear localization signals for nonviral gene transfer systems. *Gene Ther.* **2002**, *9(3)*, 157–167.

89 Escriou, V., Carriere, M., Scherman, D., Wils, P., NLS bioconjugates for targeting therapeutic genes to the nucleus. *Adv. Drug. Deliv. Rev.* **2003**, *55(2)*, 295–306.

90 Hebert, E., Improvement of exogenous DNA nuclear importation by nuclear localization signal-bearing vectors: a promising way for non-viral gene therapy? *Biol. Cell* **2003**, *95(2)*, 59–68.

91 Wells, D. J., Gene therapy progress and prospects: electroporation and other physical methods. *Gene Ther.* **2004**, *11(18)*, 1363–1369.

92 Olivares, E. C., Calos, M. P., Phage C31 integrase-mediated site-specic integration for gene therapy. *Gene Therapy and Regulation* **2003**, *2(2)*, 103–120.

93 Ogris, M., Steinlein, P., Kursa, M., Mechtler, K., Kircheis, R., Wagner, E., The size of DNA/transferrin-PEI complexes is an important factor for gene expression in cultured cells. *Gene Ther.* **1998**, *5(10)*, 1425–1433.

94 Luo, D., Saltzman, W. M., Enhancement of transfection by physical concentration of DNA at the cell surface. *Nat. Biotechnol.* **2000**, *18(8)*, 893–895.

95 Bunnell, B. A., Muul, L. M., Donahue, R. E., Blaese, R. M., Morgan, R. A., High-Efficiency Retroviral-Mediated Gene Transfer Into Human And Nonhuman Primate Peripheral Blood Lymphocytes. *Proc. Natl. Acad. Sci. USA* **1995**, *92(17)*, 7739–7743.

96 Boussif, O., Zanta, M. A., Behr, J. P., Optimized galenics improve *in vitro* gene transfer with cationic molecules up to 1000-fold. *Gene Ther.* **1996**, *3(12)*, 1074–1080.

97 O'Doherty, U., Swiggard, W. J., Malim, M. H., Human immunodeficiency virus type 1 spinoculation enhances infection through virus binding. *J. Virol.* **2000**, *74(21)*, 10074–10080.

98 Huth, S., Lausier, J., Gersting, S. W., Rudolph, C., Plank, C., Welsch, U., Rosenecker, J., Insights into the mechanism of magnetofection using PEI-based magnetofectins for gene transfer. *J. Gene Med.* **2004**, *6(8)*, 923–936.

99 Plank, C., Schillinger, U., Scherer, F., Bergemann, C., Remy, J. S., Krotz, F., Anton, M., Lausier, J., Rosenecker, J., The magnetofection method: using magnetic force to enhance gene delivery. *Biol. Chem.* **2003**, *384(5)*, 737–747.

100 Chuck, A. S., Palsson, B. O., Consistent and High Rates Of Gene Transfer Can Be Obtained Using Flow-Through Transduction Over a Wide Range Of Retroviral Titers. *Human Gene Therapy* **1996**, *7(6)*, 743–750.

101 Williams, A. R., Bao, S., Miller, D. L., Filtroporation: A simple, reliable technique for transfection and macromolecular loading of cells in suspension. *Biotechnology and Bioengineering* **1999**, *65(3)*, 341–346.

102 Liu, D., Knapp, J. E., Hydrodynamics-based gene delivery. *Curr. Opin. Mol. Ther.* **2001**, *3(2)*, 192–197.

103 Isner, J. M., Myocardial gene therapy. *Nature* **2002**, *415(6868)*, 234–239.

104 Herweijer, H., Wolff, J. A., Progress and prospects: naked DNA gene transfer and therapy. *Gene Ther.* **2003**, *10(6)*, 453–458.

105 Hodges, B. L., Scheule, R. K., Hydrodynamic delivery of DNA. *Expert Opin. Biol. Ther.* **2003**, *3(6)*, 911–918.

106 Koshkina, N. V., Agoulnik, I. Y., Melton, S. L., Densmore, C. L., Knight, V., Biodistribution and pharmacokinetics of aerosol and intravenously administered DNA-polyethyleneimine complexes: optimization of pulmonary delivery and retention. *Mol. Ther.* **2003**, *8(2)*, 249–254.

107 Rudolph, C., Ortiz, A., Schillinger, U., Jauernig, J., Plank, C., Rosenecker, J., Methodological optimization of polyethylenimine (PEI)-based gene delivery to the lungs of mice via aerosol application. *J. Gene Med.* **2004**, *7(1)*, 59–66.

108 Klein, T. M., Arentzen, R., Lewis, P. A., Fitzpatrick-McElligott, S., Transformation of microbes, plants and animals by particle bombardment. *Biotechnology (N Y)* **1992**, *10(3)*, 286–291.

109 Bonadio, J., Genetic approaches to tissue repair. *Ann. N Y Acad. Sci.* **2002**, *961*, 58–60.

110 Sharif, F., Daly, K., Crowley, J., O'Brien, T., Current status of catheter- and stent-based gene therapy. *Cardiovasc. Res.* **2004**, *64(2)*, 208–216.

111 Eliaz, R. E., Szoka, F. C., Jr. Robust and prolonged gene expression from injectable polymeric implants. *Gene Ther.* **2002**, *9(18)*, 1230–1237.

112 Christman, K. L., Fang, Q., Yee, M. S., Johnson, K. R., Sievers, R. E., Lee, R. J., Enhanced neovasculature formation in ischemic myocardium following delivery of pleiotrophin plasmid in a biopolymer. *Biomaterials* **2005**, *26(10)*, 1139–1144.

113 Scherer, F., Schillinger, U., Putz, U., Stemberger, A., Plank, C., Nonviral vector loaded collagen sponges for sustained gene delivery *in vitro* and *in vivo*. *J. Gene Med.* **2002**, *4(6)*, 634–643.

114 Bonadio, J., Smiley, E., Patil, P., Goldstein, S., Localized, direct plasmid gene delivery *in vivo*: prolonged therapy results in reproducible tissue regeneration. *Nat. Med.* **1999**, *5(7)*, 753–759.

115 Goldstein, S. A., Bonadio, J., Potential role for direct gene transfer in the enhancement of fracture healing. *Clin. Orthop.* **1998** (355 Suppl.), S154–S162.

116 Goldstein, S. A., *In vivo* nonviral delivery factors to enhance bone repair. *Clin. Orthop.* **2000** (379 Suppl.), S113–S119.

117 Kyriakides, T. R., Hartzel, T., Huynh, G., Bornstein, P., Regulation of angiogenesis and matrix remodeling by localized, matrix- mediated antisense gene delivery. *Mol. Ther.* **2001**, *3(6)*, 842–849.

118 Tyrone, J. W., Mogford, J. E., Chandler, L. A., Ma, C., Xia, Y., Pierce, G. F., Mustoe, T. A., Collagen-embedded platelet-derived growth factor DNA plasmid promotes wound healing in a dermal ulcer model. *J. Surg. Res.* **2000**, *93(2)*, 230–236.

119 Berry, M., Gonzalez, A. M., Clarke, W., Greenlees, L., Barrett, L., Tsang, W., Seymour, L., Bonadio, J., Logan, A., Baird, A., Sustained effects of gene-activated matrices after CNS injury. *Mol. Cell. Neurosci.* **2001**, *17(4)*, 706–716.

120 Pakkanen, T. M., Laitinen, M., Hippelainen, M., Hiltunen, M. O., Alhava, E., Yla-Herttuala, S., Periadventitial lacZ gene transfer to pig carotid arteries using a biodegradable collagen collar or a wrap of collagen sheet with adenoviruses and plasmid-liposome complexes. *J. Gene Med.* **2000**, *2(1)*, 52–60.

121 Chandler, L. A., Gu, D. L., Ma, C., Gonzalez, A. M., Doukas, J., Nguyen, T., Pierce, G. F., Phillips, M. L., Matrix-enabled gene transfer for cutaneous wound repair. *Wound Repair Regen.* **2000**, *8(6)*, 473–479.

122 Gu, D. L., Nguyen, T., Gonzalez, A. M., Printz, M. A., Pierce, G. F., Sosnowski, B. A., Phillips, M. L., Chandler, L. A., Adenovirus encoding human platelet-derived growth factor-B delivered in collagen exhibits safety, biodistribution, and immunogenicity profiles favorable for clinical use. *Mol. Ther.* **2004**, *9(5)*, 699–711.

123 Doukas, J., Chandler, L. A., Gonzalez, A. M., Gu, D., Hoganson, D. K., Ma, C., Nguyen, T., Printz, M. A., Nesbit, M., Herlyn, M., Crombleholme, T. M., Aukerman, S. L., Sosnowski, B. A., Pierce, G. F., Matrix immobilization enhances the tissue repair activity of growth factor gene therapy vectors. *Hum. Gene Ther.* **2001**, *12(7)*, 783–798.

124 Takahashi, A., Palmer-Opolski, M., Smith, R. C., Walsh, K., Transgene delivery of plasmid DNA to smooth muscle cells and macrophages from a biostable polymer-coated stent. *Gene Ther.* **2003**, *10(17)*, 1471–1478.

125 Klugherz, B. D., Jones, P. L., Cui, X., Chen, W., Meneveau, N. F., DeFelice, S., Connolly, J., Wilensky, R. L., Levy, R. J., Gene delivery from a DNA controlled-release stent in porcine coronary arteries. *Nat. Biotechnol.* **2000**, *18(11)*, 1181–1184.

126 Somiari, S., Glasspool-Malone, J., Drabick, J. J., Gilbert, R. A., Heller, R., Jaroszeski, M. J., Malone, R. W., Theory and *in vivo* application of electroporative gene delivery. *Mol. Ther.* **2000**, *2(3)*, 178–187.

127 Bloquel, C., Fabre, E., Bureau, M. F., Scherman, D., Plasmid DNA electrotransfer for intracellular and secreted proteins expression:

new methodological developments and applications. *J. Gene Med.* **2004**, 6 Suppl. 1, S11–S23.

128 Reynolds, P. N., Nicklin, S. A., Kaliberova, L., Boatman, B. G., Grizzle, W. E., Balyasnikova, I. V., Baker, A. H., Danilov, S. M., Curiel, D. T., Combined transductional and transcriptional targeting improves the specificity of transgene expression *in vivo*. *Nat. Biotechnol.* **2001**, *19(9)*, 838–842.

129 Dhawan, J., Rando, T. A., Elson, S. L., Bujard, H., Blau, H. M., Tetracycline-Regulated Gene Expression Following Direct Gene Transfer Into Mouse Skeletal Muscle. *Somatic Cell And Molecular Genetics* **1995**, *21(4)*, 233–240.

130 Toniatti, C., Bujard, H., Cortese, R., Ciliberto, G., Gene therapy progress and prospects: transcription regulatory systems. *Gene Ther.* **2004**, *11(8)*, 649–657.

131 Goodman, R., Blank, M., Insights into electromagnetic interaction mechanisms. *J. Cell Physiol.* **2002**, *192(1)*, 16–22.

132 Guilhon, E., Voisin, P., de Zwart, J. A., Quesson, B., Salomir, R., Maurange, C., Bouchaud, V., Smirnov, P., de Verneuil, H., Vekris, A., Canioni, P., Moonen, C. T., Spatial and temporal control of transgene expression *in vivo* using a heat-sensitive promoter and MRI-guided focused ultrasound. *J. Gene Med.* **2003**, *5(4)*, 333–342.

133 Ito, A., Shinkai, M., Honda, H., Kobayashi, T., Heat-inducible TNF-alpha gene therapy combined with hyperthermia using magnetic nanoparticles as a novel tumor-targeted therapy. Cancer *Gene Ther.* **2001**, *8(9)*, 649–654.

134 Binley, K., Askham, Z., Martin, L., Spearman, H., Day, D., Kingsman, S., Naylor, S., Hypoxia-mediated tumour targeting. *Gene Ther.* **2003**, *10(7)*, 540–549.

135 Stacy, D. R., Lu, B., Hallahan, D. E., Radiation-guided drug delivery systems. *Expert Rev. Anticancer Ther.* **2004**, *4(2)*, 283–288.

136 Li, Z., Ning, W., Wang, J., Choi, A., Lee, P. Y., Tyagi, P., Huang, L., Controlled gene delivery system based on thermosensitive biodegradable hydrogel. *Pharm. Res.* **2003**, *20(6)*, 884–888.

137 Klibanov, A. L., Ultrasound Contrast Agents: Development of the Field and Current Status. *Topics in Current Chemistry* **2002**, *222*, 73–106.

138 Bekeredjian, R., Chen, S., Frenkel, P. A., Grayburn, P. A., Shohet, R. V., Ultrasound-targeted microbubble destruction can repeatedly direct highly specific plasmid expression to the heart. *Circulation* **2003**, *108(8)*, 1022–1026.

139 Unger, E. C., Porter, T., Culp, W., Labell, R., Matsunaga, T., Zutshi, R., Therapeutic applications of lipid-coated microbubbles. *Adv. Drug. Deliv. Rev.* **2004**, *56(9)*, 1291–1314.

140 Tsutsui, J. M., Xie, F., Porter, R. T., The use of microbubbles to target drug delivery. *Cardiovasc. Ultrasound* **2004**, *2(1)*, 23.

141 Gorlich, D., Kutay, U., Transport between the cell nucleus and the cytoplasm. *Annu. Rev. Cell Dev. Biol.* **1999**, *15*, 607–660.

142 Keminer, O., Peters, R., Permeability of single nuclear pores. *Biophys. J.* **1999**, *77(1)*, 217–228.

143 Lewin, M., Carlesso, N., Tung, C. H., Tang, X. W., Cory, D., Scadden, D. T., Weissleder, R., Tat peptide-derivatized magnetic nanoparticles allow *in vivo* tracking and recovery of progenitor cells. *Nat. Biotechnol.* **2000**, *18(4)*, 410–414.

144 Capecchi, M. R., High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. *Cell* **1980**, *22(2 Pt 2)*, 479–488.

145 Hagstrom, J. E., Ludtke, J. J., Bassik, M. C., Sebestyen, M. G., Adam, S. A., Wolff, J. A., Nuclear import of DNA in digitonin-permeabilized cells. *Journal Of Cell Science* **1997**, 110(PT18), 2323–2331.

146 Jans, D. A., Chan, C. K., Huebner, S., Signals mediating nuclear targeting and their regulation: application in drug delivery. *Med. Res. Rev.* **1998**, *18(4)*, 189–223.

147 Ciolina, C., Byk, G., Blanche, F., Thuillier, V., Scherman, D., Wils, P., Coupling of nuclear localization signals to plasmid DNA and specific interaction of the conjugates with importin alpha. *Bioconjug. Chem.* **1999**, *10(1)*, 49–55.

148 Sebestyen, M. G., Ludtke, J. J., Bassik, M. C., Zhang, G., Budker, V., Lukhtanov, E. A., Hagstrom, J. E., Wolff, J. A., DNA vector chemistry: the covalent attachment of signal peptides to plasmid DNA. *Nat. Biotechnol.* **1998**, *16(1)*, 80–85.

149 Neves, C., Escriou, V., Byk, G., Scherman, D., Wils, P., Intracellular fate and nuclear targeting of plasmid DNA. *Cell Biol. Toxicol.* **1999**, *15(3)*, 193–202.

150 Zanta, M. A., Belguise-Valladier, P., Behr, J. P., Gene delivery: A single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus. *Proc. Natl. Acad. Sci. USA* **1999**, *96(1)*, 91–96.

151 van der Aa, M. A., Koning, G. A., d'Oliveira, C., Oosting, R. S., Wilschut, K. J., Hennink, W. E., Crommelin, D. J., An NLS peptide covalently linked to linear DNA does not enhance transfection efficiency of cationic polymer based gene delivery systems. *J. Gene Med.* **2004**.

152 Branden, L. J., Mohamed, A. J., Smith, C. I., A peptide nucleic acid-nuclear localization signal fusion that mediates nuclear transport of DNA. *Nat. Biotechnol.* **1999**, *17(8)*, 784–787.

153 Subramanian, A., Ranganathan, P., Diamond, S. L., Nuclear targeting peptide scaffolds for lipofection of nondividing mammalian cells. *Nat. Biotechnol.* **1999**, *17(9)*, 873–877.

154 Singh, D., Bisland, S. K., Kawamura, K., Gariepy, J., Peptide-based intracellular shuttle able to facilitate gene transfer in mammalian cells. *Bioconj. Chem.* **1999**, *10(5)*, 745–754.

155 Chan, C. K., Jans, D. A., Enhancement of polylysine-mediated transferrinfection by nuclear localization sequences: polylysine does not function as a nuclear localization sequence. *Hum. Gene Ther.* **1999**, *10(10)*, 1695–1702.

156 Collas, P., Alestrom, P., Nuclear localization signals enhance germline transmission of a transgene in zebrafish. *Transgenic Res.* **1998**, *7(4)*, 303–309.

157 Ritter, W., Plank, C., Lausier, J., Rudolph, C., Zink, D., Reinhardt, D., Rosenecker J. A novel transfecting peptide comprising a tetrameric nuclear localization sequence. *J. Mol. Med.* **2003**, *81(11)*, 708–717.

158 Morris, M. C., Vidal, P., Chaloin, L., Heitz, F., Divita G. A new peptide vector for efficient delivery of oligonucleotides into mammalian cells. *Nucleic Acids Res.* **1997**, *25(14)*, 2730–2736.

159 Simeoni, F., Morris, M. C., Heitz, F., Divita, G., Insight into the mechanism of the peptide-based gene delivery system MPG: implications for delivery of siRNA into mammalian cells. *Nucleic Acids Res.* **2003**, *31(11)*, 2717–2724.

160 Morris, K. V., Chan, S. W., Jacobsen, S. E., Looney, D. J., Small interfering RNA-induced transcriptional gene silencing in human cells. *Science* **2004**, *305(5688)*, 1289–1292.

161 Rudolph, C., Plank, C., Lausier, J., Schillinger, U., Muller, R. H., Rosenecker, J., Oligomers of the Arginine-rich Motif of the HIV-1 TAT Protein Are Capable of Transferring Plasmid DNA into Cells. *J. Biol. Chem.* **2003**, *278(13)*, 11411–11418.

162 Truant, R., Cullen, B. R., The arginine-rich domains present in human immunodeficiency virus type 1 Tat and Rev function as direct importin beta-dependent nuclear localization signals. *Mol. Cell Biol.* **1999**, *19(2)*, 1210–1217.

163 Torchilin, V. P., Rammohan, R., Weissig, V., Levchenko, T. S., TAT peptide on the surface of liposomes affords their efficient intracellular delivery even at low temperature and in the presence of metabolic inhibitors. *Proc. Natl. Acad. Sci. USA* **2001**, *98(15)*, 8786–8791.

164 Eguchi, A., Akuta, T., Okuyama, H., Senda, T., Yokoi, H., Inokuchi, H., Fujita, S., Hayakawa, T., Takeda, K., Hasegawa, M., Nakanishi, M., Protein transduction domain of HIV-1 Tat protein promotes efficient delivery of DNA into mammalian cells. *J. Biol. Chem.* **2001**, *276(28)*, 26204–26210.

165 Wilson, G. L., Dean, B. S., Wang, G., Dean, D. A., Nuclear import of plasmid DNA in digitonin-permeabilized cells requires both cytoplasmic factors and specific DNA sequences. *J. Biol. Chem.* **1999**, *274(31)*, 22025–22032.

166 Dean, D. A., Dean, B. S., Muller, S., Smith, L. C., Sequence requirements for plasmid nuclear import. *Exp. Cell Res.* **1999**, *253(2)*, 713–722.

167 Dean, D. A., Import of plasmid DNA into the nucleus is sequence specific. *Exp. Cell Res.* **1997**, *230(2)*, 293–302.

168 Li, S., MacLaughlin, F. C., Fewell, J. G., Gondo, M., Wang, J., Nicol, F., Dean, D. A., Smith, L. C., Muscle-specific enhancement of gene expression by incorporation of SV40 enhancer in the expression plasmid. *Gene Ther.* **2001**, *8(6)*, 494–497.

169 Mesika, A., Grigoreva, I., Zohar, M., Reich, Z., A regulated, NFkappaB-assisted import of plasmid DNA into mammalian cell nuclei. *Mol. Ther.* **2001**, *3(5 Pt 1)*, 653–657.

170 Nishikawa, M., Kuramoto, T., Okabe, T., Takakura, Y., Hashida, M., *Mol. Ther.* **2003**, *7(167)*, Abstract No. 423.

171 Zou, S. M., Erbacher, P., Remy, J. S., Behr, J. P., Systemic linear polyethylenimine (L-PEI)-mediated gene delivery in the mouse. *J. Gene Med.* **2000**, *2(2)*, 128–134.

172 Barron, L. G., Gagne, L., Szoka, F. C., Jr. Lipoplex-mediated gene delivery to the lung occurs within 60 minutes of intravenous administration. *Hum. Gene Ther.* **1999**, *10(10)*, 1683–1694.

173 Oh, Y. K., Kim, J. P., Yoon, H., Kim, J. M., Yang, J. S., Kim, C. K., Prolonged organ retention and safety of plasmid DNA administered in polyethylenimine complexes. *Gene Ther.* **2001**, *8(20)*, 1587–1592.

174 Kircheis, R., Schuller, S., Brunner, S., Ogris, M., Heider, K. H., Zauner, W., Wagner, E., Polycation-based DNA complexes for tumor-targeted gene delivery *in vivo*. *J. Gene Med.* **1999**, *1(2)*, 111–120.

175 Ogris, M., Brunner, S., Schuller, S., Kircheis, R., Wagner, E., PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. *Gene Ther.* **1999**, *6(4)*, 595–605.

176 Hood, J. D., Bednarski, M., Frausto, R., Guccione, S., Reisfeld, R. A., Xiang, R., Cheresh, D. A., Tumor regression by targeted gene delivery to the neovasculature. *Science* **2002**, *296(5577)*, 2404–2407.

177 Darji, A., Guzman, C. A., Gerstel, B., Wachholz, P., Timmis, K. N., Wehland, J., Chakraborty, T., Weiss, S., Oral somatic transgene vaccination using attenuated S. typhimurium. *Cell* **1997**, *91(6)*, 765–775.

178 Niethammer, A. G., Xiang, R., Ruehlmann, J. M., Lode, H. N., Dolman, C. S., Gillies, S. D., Reisfeld, R. A., Targeted interleukin 2 therapy enhances protective immunity induced by an autologous oral DNA vaccine against murine melanoma. *Cancer Res.* **2001**, *61(16)*, 6178–6184.

179 Niethammer, A. G., Xiang, R., Becker, J. C., Wodrich, H., Pertl, U., Karsten, G., Eliceiri, B. P., Reisfeld, R. A., A DNA vaccine against VEGF receptor 2 prevents effective angiogenesis and inhibits tumor growth. *Nat. Med.* **2002**, *4*, 4.

180 Reisfeld, R. A., Niethammer, A. G., Luo, Y., Xiang, R., DNA vaccines suppress tumor growth and metastases by the induction of anti-angiogenesis. *Immunol. Rev.* **2004**, *199*, 181–190.

181 Fu, G. F., Li, X., Hou, Y. Y., Fan, Y. R., Liu, W. H., Xu, G. X., Bifidobacterium longum as an oral delivery system of endostatin for gene therapy on solid liver cancer. *Cancer Gene Ther.* **2005**, *12(2)*, 133–140.

182 Zhao, J., Lou, Y., Pinczewski, J., Malkevitch, N., Aldrich, K., Kalyanaraman, V. S., Venzon, D., Peng, B., Patterson, L. J., Edghill-Smith, Y., Woodward, R., Pavlakis, G. N., Robert-Guroff, M., Boosting of SIV-specific immune responses in rhesus macaques by repeated administration of Ad5hr-SIVenv/rev and Ad5hr-SIVgag recombinants. *Vaccine* **2003**, *21(25–26)*, 4022–4035.

183 Fayad, R., Zhang, H., Quinn, D., Huang, Y., Qiao, L., Oral administration with papillomavirus pseudovirus encoding IL-2 fully restores mucosal and

systemic immune responses to vaccinations in aged mice. *J. Immunol.* **2004**, *173(4)*, 2692–2698.

184 Kai, E., Ochiya T. A method for oral DNA delivery with N-acetylated chitosan. *Pharm. Res.* **2004**, *21(5)*, 838–843.

185 Hejazi, R., Amiji, M., Chitosan-based gastrointestinal delivery systems. *J. Control Release* **2003**, *89(2)*, 151–165.

186 Roy, K., Mao, H. Q., Huang, S. K., Leong, K. W., Oral gene delivery with chitosan–DNA nanoparticles generates immunologic protection in a murine model of peanut allergy. *Nat. Med.* **1999**, *5(4)*, 387–391.

187 Jones, D. H., Corris, S., McDonald, S., Clegg, J. C., Farrar, G. H., Poly(dl-lactide-co-glycolide)-encapsulated plasmid DNA elicits systemic and mucosal antibody responses to encoded protein after oral administration. *Vaccine* **1997**, *15(8)*, 814–817.

188 Chen, S. C., Jones, D. H., Fynan, E. F., Farrar, G. H., Clegg, J. C., Greenberg, H. B., Herrmann, J. E., Protective immunity induced by oral immunization with a rotavirus DNA vaccine encapsulated in microparticles. *J. Virol.* **1998**, *72(7)*, 5757–5761.

189 Mittal, S. K., Aggarwal, N., Sailaja, G., van Olphen, A., HogenEsch, H., North, A., Hays, J., Moffatt, S., Immunization with DNA, adenovirus or both in biodegradable alginate microspheres: effect of route of inoculation on immune response. *Vaccine* **2000**, *19(2–3)*, 253–263.

190 Moore, R. A., Walcott, S., White, K. L., Anderson, D. M., Jain, S., Lloyd, A., Topley, P., Thomsen, L., Gough, G. W., Stanley, M. A., Therapeutic immunisation with COPV early genes by epithelial DNA delivery. *Virology* **2003**, *314(2)*, 630–635.

191 Chang, S. F., Chang, H. Y., Tong, Y. C., Chen, S. H., Hsaio, F. C., Lu, S. C., Liaw, J., Nonionic polymeric micelles for oral gene delivery *in vivo*. *Hum. Gene Ther.* **2004**, *15(5)*, 481–493.

192 Howard, K. A., Li, X. W., Somavarapu, S., Singh, J., Green, N., Atuah, K. N., Ozsoy, Y., Seymour, L. W., Alpar, H. O., Formulation of a microparticle carrier for oral polyplex-based DNA vaccines. *Biochim. Biophys. Acta* **2004**, *1674(2)*, 149–157.

193 Takamura, S., Niikura, M., Li, T. C., Takeda, N., Kusagawa, S., Takebe, Y., Miyamura, T., Yasutomi, Y., DNA vaccine-encapsulated virus-like particles derived from an orally transmissible virus stimulate mucosal and systemic immune responses by oral administration. *Gene Ther.* **2004**, *11(7)*, 628–635.

194 Gander, B., Trends in particulate antigen and DNA delivery systems for vaccines. *Adv. Drug. Deliv. Rev.* **2005**, *57(3)*, 321–323.

195 Scherer, F., Anton, M., Schillinger, U., Henke, J., Bergemann, C., Kruger, A., Gansbacher, B., Plank, C., Magnetofection: enhancing and targeting gene delivery by magnetic force *in vitro* and *in vivo*. *Gene Ther.* **2002**, *9(2)*, 102–109.

196 Alksne, J. F., Fingerhut, A. G., Magnetically controlled metallic thrombosis of intracranial aneurysms. A preliminary report. *Bull. Los Angeles Neurol. Soc.* **1965**, *30(3)*, 153–155.

197 Fingerhut, A. G., Alksne, J. F., Thrombosis of intracranial aneurysms. An experimental approach utilizing magnetically controlled iron particles. *Radiology* **1966**, *86(2)*, 342–343.

198 Alksne, J. F., Fingerhut, A. G., Rand, R. W., Magnetically controlled focal intravascular thrombosis in dogs. *J. Neurosurg.* **1966**, *25(5)*, 516–525.

199 Meyers, P. H., Nice, C. M., Jr., Meckstroth, G. R., Becker, H. C., Moser, P. J., Goldstein, M., Pathologic studies following magnetic control of metallic iron particles in the lymphatic and vascular system of dogs as a contrast and isotopic agent. *Am. J. Roentgenol. Radium Ther. Nucl. Med.* **1966**, *96(4)*, 913–921.

200 Widder, K. J., Senyei, A. E., Scarpelli, G. D., Magnetic microspheres: a model system of site specific drug delivery *in vivo*. *Proc. Soc. Exp. Biol. Med.* **1978**, *158(2)*, 141–146.

201 Fahlvik, A. K., Klaveness, J., Stark, D. D., Iron oxides as MR imaging contrast agents. *J. Magn. Reson. Imaging* **1993**, *3(1)*, 187–194.

202 Carlin, R. L., Magnetochemistry. Heidelberg: Springer **1986**.

203 Weiss, A., Witte, H., Magnetochemie: Grundlagen und Anwendungen. Weinheim: Wiley/VCH **1997**.

204 Senyei, A. E., Reich, S. D., Gonczy, C., Widder, K. J., *In vivo* kinetics of magnetically targeted low-dose doxorubicin. *J. Pharm. Sci.* **1981**, *70(4)*, 389–391.

205 Widder, K. J., Morris, R. M., Poore, G., Howard, D. P., Jr., Senyei, A. E., Tumor remission in Yoshida sarcoma-bearing rats by selective targeting of magnetic albumin microspheres containing doxorubicin. *Proc. Natl. Acad. Sci. USA* **1981**, *78(1)*, 579–581.

206 Widder, K. J., Morris, R. M., Poore, G. A., Howard, D. P., Senyei, A. E., Selective targeting of magnetic albumin microspheres containing low-dose doxorubicin: total remission in Yoshida sarcoma-bearing rats. *Eur. J. Cancer Clin. Oncol.* **1983**, *19(1)*, 135–139.

207 Gupta, P. K., Hung, C. T., Comparative disposition of adriamycin delivered via magnetic albumin microspheres in presence and absence of magnetic field in rats. *Life Sci.* **1990**, *46(7)*, 471–479.

208 Gupta, P. K., Hung, C. T., Effect of carrier dose on the multiple tissue disposition of doxorubicin hydrochloride administered via magnetic albumin microspheres in rats. *J. Pharm. Sci.* **1989**, *78(9)*, 745–748.

209 Gupta, P. K., Hung, C. T., Rao, N. S., Ultrastructural disposition of adriamycin-associated magnetic albumin microspheres in rats. *J. Pharm. Sci.* **1989**, *78(4)*, 290–294.

210 Gupta, P. K., Hung, C. T., Magnetically controlled targeted micro-carrier systems. *Life Sci.* **1989**, *44(3)*, 175–186.

211 Gupta, P. K., Hung, C. T., Targeted delivery of low dose doxorubicin hydrochloride administered via magnetic albumin microspheres in rats. *J. Microencapsul.* **1990**, *7(1)*, 85–94.

212 Kato, T., Nemoto, R., Mori, H., Unno, K., Goto, A., Homma M. [An approach to magnetically controlled cancer chemotherapy.

I. Preparation and properties of ferromagnetic mitomycin C microcapsules (author's transl.)]. *Nippon Gan Chiryo Gakkai Shi* **1980**, *15(5)*, 876–880.

213 Kato, T., Nemoto, R., Mori, H., Abe, R., Unno, K., Goto, A., Murota, H., Harada, M., Homma, M., Magnetic microcapsules for targeted delivery of anticancer drugs. *Appl. Biochem. Biotechnol.* **1984**, *10*, 199–211.

214 Lubbe, A. S., Bergemann, C., Huhnt, W., Fricke, T., Riess, H., Brock, J. W., Huhn, D., Preclinical experiences with magnetic drug targeting: tolerance and efficacy. *Cancer Res.* **1996**, *56(20)*, 4694–4701.

215 Lubbe, A. S., Bergemann, C., Riess, H., Schriever, F., Reichardt, P., Possinger, K., Matthias, M., Dorken, B., Herrmann, F., Gurtler, R., Hohenberger, P., Haas, N., Sohr, R., Sander, B., Lemke, A. J., Ohlendorf, D., Huhnt, W., Huhn, D., Clinical experiences with magnetic drug targeting: a phase I study with 4′-epidoxorubicin in 14 patients with advanced solid tumors. *Cancer Res.* **1996**, *56(20)*, 4686–4693.

216 Lübbe, A. S., Bergemann, C., Selected preclinical and first clinical experiences with magnetically targeted 4-epidoxorubicin in patients with advanced solid tumors. In: Häfeli, U., Schütt, W., Teller, J., Zborowski, M. (Eds.), *Scientific and Clinical Applications of Magnetic Drug Carriers*. New York, London: Plenum Press **1997**.

217 Lemke, A. J., Senfft von Pilsach, M. I., Lubbe, A., Bergemann, C., Riess, H., Felix, R., MRI after magnetic drug targeting in patients with advanced solid malignant tumors. *Eur. Radiol.* **2004**, *14(11)*, 1949–1955.

218 Goodwin, S. C., Bittner, C. A., Peterson, C. L., Wong, G., Single-dose toxicity study of hepatic intra-arterial infusion of doxorubicin coupled to a novel magnetically targeted drug carrier. *Toxicol. Sci.* **2001**, *60(1)*, 177–183.

219 Goodwin, S. C., Peterson, C., Hoh, C., Bittner, C. A., Targeting and retention of magnetic targeted carriers (MTCs) enhancing intra-arterial chemotherapy. *J. Magn. Magn. Mat.* **1999**, *194*, 132–139.

220 Rudge, S. R., Kurtz, T. L., Vessely, C. R., Catterall, L. G., Williamson, D. L., Preparation, characterization, and performance of magnetic iron-carbon composite microparticles for chemotherapy. *Biomaterials* **2000**, *21(14)*, 1411–1420.

221 Rudge, S., Peterson, C., Vessely, C., Koda, J., Stevens, S., Catterall, L., Adsorption and desorption of chemotherapeutic drugs from a magnetically targeted carrier (MTC). *J. Control Release* **2001**, *74(1–3)*, 335–340.

222 Johnson, J., Kent, T., Koda, J., Peterson, C., Rudge, S., Tapolsky, G., The MTC technology: A platform technology for the site-specific delivery of pharmaceutical agents. In: *4th International Conference on the Scientific and Clinical Applications of Magnetic Carriers, 2002*. Tallahassee: European Cells and Materials **2002**, 12–15.

223 Widder, K. J., Marino, P. A., Morris, R. M., Howard, D. P., Poore, G. A., Senyei, A. E., Selective targeting of magnetic albumin microspheres to the Yoshida sarcoma: ultrastructural evaluation of microsphere disposition. *Eur. J. Cancer Clin. Oncol.* **1983**, *19(1)*, 141–147.

224 Plank, C., Bergemann, C., Method for transfecting cells using a magnetic field. European patent application WO 0200870, publication date 2002. Requested patent No. EP 1297169.

225 Haim, H., Steiner, I., Panet, A., Synchronized infection of cell cultures by magnetically controlled virus. *J. Virol.* **2005**, *79(1)*, 622–625.

226 Wu, J., Bratko, D., Prausnitz, J. M., Interaction between like-charged colloidal spheres in electrolyte solutions. *Proc. Natl. Acad. Sci. USA* **1998**, *95(26)*, 15169–15172.

227 Hughes, C., Galea-Lauri, J., Farzaneh, F., Darling, D., Streptavidin paramagnetic particles provide a choice of three affinity- based capture and magnetic concentration strategies for retroviral vectors. *Mol. Ther.* **2001**, *3(4)*, 623–630.

228 Mah, C., Fraites T. J. J., Zolotukhin, I., Song, S., Flotte, T. R., Jon Dobson, Batich, C., Byrne, B. J., Improved Method of Recombinant AAV2 Delivery for Systemic Targeted Gene Therapy. *Mol. Ther.* **2002**, *6(1)*, 106–112.

229 Pandori, M. W., Hobson, D. A., Sano, T., Adenovirus-Microbead Conjugates Prossess Enhanced Infectivity: A New Strategy to Localized Gene Delivery. *Virology* **2002**, *299*, 204–212.

230 Raty, J. K., Airenne, K. J., Marttila, A. T., Marjomaki, V., Hytonen, V. P., Lehtolainen, P., Laitinen, O. H., Mahonen, A. J., Kulomaa, M. S., Yla-Herttuala, S., Enhanced gene delivery by avidin-displaying baculovirus. *Mol. Ther.* **2004**, *9(2)*, 282–291.

231 Krotz, F., Wit, C., Sohn, H. Y., Zahler, S., Gloe, T., Pohl, U., Plank, C., Magnetofection-A highly efficient tool for antisense oligonucleotide delivery *in vitro* and *in vivo*. *Mol. Ther.* **2003**, *7(5)*, 700–710.

232 Krotz, F., Sohn, H. Y., Gloe, T., Plank, C., Pohl, U., Magnetofection Potentiates Gene Delivery to Cultured Endothelial Cells. *J. Vasc. Res.* **2003**, *40(5)*, 425–434.

233 Gersting, S. W., Schillinger, U., Lausier, J., Nicklaus, P., Rudolph, C., Plank, C., Reinhardt, D., Rosenecker, J., Gene delivery to respiratory epithelial cells by magnetofection. *J. Gene Med.* **2004**, *6(8)*, 913–922.

234 Schillinger, U., Brill, T., Rudolph, C., Huth, S., Gersting, S., Krotz, F., Hirschberger, J., Bergemann, C., Plank, C., Advances in Magnetofection – magnetically guided nucleic acid delivery. *J. Magn. Mat.* **2005**, *293*, 501–508.

235 Couto, S. S., Griffey, S. M., Duarte, P. C., Madewell, B. R., Feline vaccine-associated fibrosarcoma: morphologic distinctions. *Vet. Pathol.* **2002**, *39(1)*, 33–41.

236 Zborowski, M., Fuh, C. B., Green, R., Sun, L., Chalmers, J. J., Analytical magnetapheresis of ferritin-labeled lymphocytes. *Anal. Chem.* **1995**, *67(20)*, 3702–3712.

237 Voltairas, P. A., Fotiadis, D. I., Michalis, L. K., Hydrodynamics of magnetic drug targeting. *J. Biomech.* **2002**, *35(6)*, 813–821.

238 Babincova, M., Babinec, P., Possibility of magnetic targeting of drugs using magnetoliposomes. *Pharmazie* **1995**, *50(12)*, 828–829.

239 Nagel, S., Theoretische und experimentelle Untersuchungen zum Magnetischen Drug Targeting. Greifswald: Ernst-Moritz-Arndt-Universität Greifswald **2004**.

240 Babincova, M., Babinec, P., Bergemann, C., High-gradient magnetic capture of ferrofluids: implications for drug targeting and tumor embolization. *Z. Naturforsch. [C]* **2001**, *56(9–10)*, 909–911.

241 Benvenisty, N., Reshef, L., Direct introduction of genes into rats and expression of the genes. *Proc. Natl. Acad. Sci. USA* **1986**, *83(24)*, 9551–9555.

242 Dubensky, T. W., Campbell, B. A., Villarreal, L. P., Direct transfection of viral and plasmid DNA into the liver or spleen of mice. *Proc. Natl. Acad. Sci. USA* **1984**, *81(23)*, 7529–7533.

243 Will, H., Cattaneo, R., Koch, H. G., Darai, G., Schaller, H., Schellekens, H., van Eerd, P. M., Deinhardt, F., Cloned HBV DNA causes hepatitis in chimpanzees. *Nature* **1982**, *299(5885)*, 740–742.

244 Budker, V., Zhang, G., Knechtle, S., Wolff, J. A., Naked DNA delivered intraportally expresses efficiently in hepatocytes. *Gene Ther.* **1996**, *3(7)*, 593–598.

245 Zhang, G., Vargo, D., Budker, V., Armstrong, N., Knechtle, S., Wolff, J. A., Expression of naked plasmid DNA injected into the afferent and efferent vessels of rodent and dog livers. *Hum. Gene Ther.* **1997**, *8(15)*, 1763–1772.

246 Liu, F., Song, Y., Liu, D., Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther.* **1999**, *6(7)*, 1258–1266.

247 Zhang, G., Budker, V., Wolff, J. A., High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. *Hum. Gene Ther.* **1999**, *10(10)*, 1735–1737.

248 Zhang, G., Gao, X., Song, Y. K., Vollmer, R., Stolz, D. B., Gasiorowski, J. Z., Dean, D. A., Liu, D., Hydroporation as the mechanism of hydrodynamic delivery. *Gene Ther.* **2004**, *11(8)*, 675–682.

249 Lewis, D. L., Hagstrom, J. E., Loomis, A. G., Wolff, J. A., Herweijer, H., Efficient delivery of siRNA for inhibition of gene expression in postnatal mice. *Nat. Genet.* **2002**, *32(1)*, 107–108.

250 McCaffrey, A. P., Meuse, L., Pham, T. T., Conklin, D. S., Hannon, G. J., Kay, M. A., RNA interference in adult mice. *Nature* **2002**, *418(6893)*, 38–39.

251 Song, E., Lee, S. K., Wang, J., Ince, N., Ouyang, N., Min, J., Chen, J., Shankar, P., Lieberman, J., RNA interference targeting Fas protects mice from fulminant hepatitis. *Nat. Med.* **2003**, *9(3)*, 347–351.

252 Hagstrom, J. E., Hegge, J., Zhang, G., Noble, M., Budker, V., Lewis, D. L., Herweijer, H., Wolff, J. A., A facile nonviral method for delivering genes and siRNAs to skeletal muscle of mammalian limbs. *Mol. Ther.* **2004**, *10(2)*, 386–398.

253 Zhang, G., Budker, V., Williams, P., Subbotin, V., Wolff, J. A., Efficient expression of naked dna delivered intraarterially to limb muscles of nonhuman primates. *Hum. Gene Ther.* **2001**, *12(4)*, 427–438.

254 Friess, W., Collagen–biomaterial for drug delivery. *Eur. J. Pharm. Biopharm.* **1998**, *45(2)*, 113–136.

255 Stemberger, A., Grimm, H., Bader, F., Rahn, H. D., Ascherl, R., Local treatment of bone and soft tissue infections with the collagen-gentamicin sponge. *Eur. J. Surg. Suppl.* **1997**, *578*, 17–26.

256 Lee, K. Y., Peters, M. C., Anderson, K. W., Mooney, D. J., Controlled growth factor release from synthetic extracellular matrices. *Nature* **2000**, *408(6815)*, 998–1000.

257 Bonadio, J., Tissue engineering via local gene delivery: update and future prospects for enhancing the technology. *Adv. Drug. Deliv. Rev.* **2000**, *44(2–3)*, 185–194.

258 Pannier, A. K., Shea, L. D., Controlled release systems for DNA delivery. *Mol. Ther.* **2004**, *10(1)*, 19–26.

259 Fang, J., Zhu, Y. Y., Smiley, E., Bonadio, J., Rouleau, J. P., Goldstein, S. A., McCauley, L. K., Davidson, B. L., Roessler, B. J., Stimulation of new bone formation by direct transfer of osteogenic plasmid genes. *Proc. Natl. Acad. Sci. USA* **1996**, *93(12)*, 5753–5758.

260 Erlebacher, A., Filvaroff, E. H., Gitelman, S. E., Derynck, R., Toward a molecular understanding of skeletal development. *Cell* **1995**, *80(3)*, 371–378.

261 Madry, H., Padera, R., Seidel, J., Langer, R., Freed, L. E., Trippel, S. B., Vunjak-Novakovic, G., Gene transfer of a human insulin-like growth factor I cDNA enhances tissue engineering of cartilage. *Hum. Gene Ther.* **2002**, *13(13)*, 1621–1630.

262 Gelse, K., von der Mark, K., Aigner, T., Park, J., Schneider, H., Articular cartilage repair by gene therapy using growth factor-producing mesenchymal cells. *Arthritis Rheum.* **2003**, *48(2)*, 430–441.

263 Tandia, B. M., Vandenbranden, M., Wattiez, R., Lakhdar, Z., Ruysschaert, J. M., Elouahabi, A., Identification of human plasma proteins that bind to cationic lipid/DNA complex and analysis of their effects on transfection efficiency: implications for intravenous gene transfer. *Mol. Ther.* **2003**, *8(2)*, 264–273.

264 Isner, J. M., Pieczek, A., Schainfeld, R., Blair, R., Haley, L., Asahara, T., Rosenfield, K., Razvi, S., Walsh, E., Symes, J. F., Clinical Evidence Of Angiogenesis After Arterial Gene Transfer Of Phvegf(165) in Patient with Ischaemic Limb. *Lancet* **1996**, *348(9024)*, 370–374.

265 Moses, J. W., Leon, M. B., Popma, J. J., Fitzgerald, P. J., Holmes, D. R., O'Shaughnessy, C., Caputo, R. P., Kereiakes, D. J., Williams, D. O., Teirstein, P. S., Jaeger, J. L., Kuntz, R. E., the SIRIUS Investigators. Sirolimus-Eluting Stents versus Standard Stents in Patients with Stenosis in a Native Coronary Artery. *N. Engl. J. Med.* **2003**, 1315–1323.

266 Fattori, R., Piva T. Drug-eluting stents in vascular intervention. *Lancet* **2003**, *361(9353)*, 247–249.

267 Gollwitzer, H., Ibrahim, K., Meyer, H., Mittelmeier, W., Busch, R., Stemberger, A., Antibacterial poly(D,L-lactic acid) coating of medical implants using a biodegradable drug delivery technology. *J. Antimicrob. Chemother.* **2003**, *51(3)*, 585–591.

268 Lucke, M., Schmidmaier, G., Sadoni, S., Wildemann, B., Schiller, R., Haas, N. P., Raschke, M., Gentamicin coating of metallic implants reduces implant-related osteomyelitis in rats. Bone **2003**, *32(5)*, 521–531.

269 Wildemann, B., Bamdad, P., Holmer, C., Haas, N. P., Raschke, M., Schmidmaier, G., Local delivery of growth factors from coated titanium plates increases osteotomy healing in rats. *Bone* **2004**, *34(5)*, 862–868.

270 Schmidmaier, G., Wildemann, B., Ostapowicz, D., Kandziora, F., Stange, R., Haas, N. P., Raschke, M., Long-term effects of local growth factor (IGF-I and TGF-beta 1) treatment on fracture healing. A safety study for using growth factors. *J. Orthop. Res.* **2004**, *22(3)*, 514–519.

271 Wildemann, B., Kandziora, F., Krummrey, G., Palasdies, N., Haas, N. P., Raschke, M., Schmidmaier, G., Local and controlled release of growth factors (combination of IGF-I and TGF-beta I, and BMP-2 alone) from a polylactide coating of titanium implants does not lead to ectopic bone formation in sheep muscle. *J. Control Release* **2004**, *95(2)*, 249–256.

272 Wildemann, B., Sander, A., Schwabe, P., Lucke, M., Stockle, U., Raschke, M., Haas, N. P., Schmidmaier, G., Short term *in vivo* biocompatibility testing of biodegradable poly(D,L-lactide)-growth factor coating for orthopaedic implants. *Biomaterials* **2005**, *26(18)*, 4035–4040.

273 Shen, H., Tan, J., Saltzman, W. M., Surface-mediated gene transfer from nanocomposites of controlled texture. *Nat. Mater.* **2004**, *3(8)*, 569–574.

274 Walter, D. H., Cejna, M., Diaz-Sandoval, L., Willis, S., Kirkwood, L., Stratford, P. W., Tietz, A. B., Kirchmair, R., Silver, M., Curry, C., Wecker, A., Yoon, Y. S., Heidenreich, R., Hanley, A., Kearney, M., Tio, F. O., Kuenzler, P., Isner, J. M., Losordo, D. W., Local gene transfer of phVEGF-2 plasmid by gene-eluting stents: an alternative strategy for inhibition of restenosis. *Circulation* **2004**, *110(1)*, 36–45.

275 Tamber, H., Johansen, P., Merkle, H. P., Gander, B., Formulation aspects of biodegradable polymeric microspheres for antigen delivery. *Adv. Drug. Deliv. Rev.* **2005**, *57(3)*, 357–376.

276 Jilek, S., Merkle, H. P., Walter, E., DNA-loaded biodegradable microparticles as vaccine delivery systems and their interaction with dendritic cells. *Adv. Drug. Deliv. Rev.* **2005**, *57(3)*, 377–390.

277 Wang, C., Ge, Q., Ting, D., Nguyen, D., Shen, H. R., Chen, J., Eisen, H. N., Heller, J., Langer, R., Putnam, D., Molecularly engineered poly(ortho ester) microspheres for enhanced delivery of DNA vaccines. *Nat. Mater.* **2004**, *3(3)*, 190–196.

278 Cui, Z., Mumper, R. J., Microparticles and nanoparticles as delivery systems for DNA vaccines. *Crit. Rev. Ther. Drug Carrier Syst.* **2003**, *20(2–3)*, 103–137.

279 Mumper, R. J., Cui, Z., Genetic immunization by jet injection of targeted pDNA-coated nanoparticles. *Methods* **2003**, *31(3)*, 255–262.

280 Trimble, C., Lin, C. T., Hung, C. F., Pai, S., Juang, J., He, L., Gillison, M., Pardoll, D., Wu, L., Wu, T. C., Comparison of the CD8+ T cell responses and antitumor effects generated by DNA vaccine administered through gene gun, biojector, and syringe. *Vaccine* **2003**, *21(25–26)*, 4036–4042.

281 Goula, D., Becker, N., Lemkine, G. F., Normandie, P., Rodrigues, J., Mantero, S., Levi, G., Demeneix, B. A., Rapid crossing of the pulmonary endothelial barrier by polyethylenimine/DNA complexes. *Gene Ther.* **2000**, *7(6)*, 499–504.

282 Goula, D., Benoist, C., Mantero, S., Merlo, G., Levi, G., Demeneix, B. A., Polyethylenimine-based intravenous delivery of transgenes to mouse lung. *Gene Ther.* **1998**, *5(9)*, 1291–1295.

283 Wightman, L., Kircheis, R., Rossler, V., Carotta, S., Ruzicka, R., Kursa, M., Wagner, E., Different behavior of branched and linear polyethylenimine for gene delivery *in vitro* and *in vivo*. *J. Gene Med.* **2001**, *3(4)*, 362–372.

284 Rudolph, C., Schillinger, U., Plank, C., Gessner, A., Nicklaus, P., Muller, R., Rosenecker, J., Nonviral gene delivery to the lung with copolymer-protected and transferrin-modified polyethylenimine. *Biochim. Biophys. Acta* **2002**, *1573(1)*, 75–83.

285 Fehrenbach, H., Alveolar epithelial type II cell: defender of the alveolus revisited. *Respir. Res.* **2001**, *2(1)*, 33–46.

286 Sharma, S., White, D., Imondi, A. R., Placke, M. E., Vail, D. M., Kris, M. G., Development of inhalational agents for oncologic use. *J. Clin. Oncol.* **2001**, *19(6)*, 1839–1847.

287 Koshkina, N. V., Waldrep, J. C., Roberts, L. E., Golunski, E., Melton, S., Knight, V., Paclitaxel liposome aerosol treatment induces inhibition of pulmonary metastases in murine renal carcinoma model. *Clin. Cancer Res.* **2001**, *7(10)*, 3258–3262.

288 Rudolph, C., Lausier, J., Naundorf, S., Muller, R. H., Rosenecker, J., *In vivo* gene delivery to the lung using polyethylenimine and fractured polyamidoamine dendrimers. *J. Gene Med.* **2000**, *2(4)*, 269–278.

289 Densmore, C. L., Orson, F. M., Xu, B., Kinsey, B. M., Waldrep, J. C., Hua, P., Bhogal, B., Knight, V., Aerosol delivery of robust polyethyleneimine–DNA complexes for gene therapy and genetic immunization. *Mol. Ther.* **2000**, *1(2)*, 180–188.

290 Gautam, A., Densmore, C. L., Waldrep, J. C., Pulmonary cytokine responses associated with PEI–DNA aerosol gene therapy. *Gene Ther.* **2001**, *8(3)*, 254–257.

291 Rudolph, C., Ortiz, A., Schillinger, U., Plank, C., Rosenecker, J., *In vivo*-gene expression of aerosolized PEI gene vector complexes is solvent-dependent. *Mol. Ther.* **2004**, 9 Suppl. 1, 193–194.

292 Babincova, M., Cicmanec, P., Altanerova, V., Altaner, C., Babinec, P., AC-magnetic field controlled drug release from magnetoliposomes: design of a method for site-specific chemotherapy. *Bioelectrochemistry* **2002**, *55(1–2)*, 17–19.

293 Babincova, M., Altanerova, V., Altaner, C., Cicmanec, P., Babinec, P., *In vivo* heating of magnetic nanoparticles in alternating magnetic field. *Med. Phys.* **2004**, *31(8)*, 2219–2221.

294 Schutt, E. G., Klein, D. H., Mattrey, R. M., Riess, J. G., Injectable microbubbles as contrast agents for diagnostic ultrasound imaging: the key role of perfluorochemicals. *Angew. Chem. Int. Ed. Engl.* **2003**, *42(28)*, 3218–3235.

295 Gramiak, R., Shah, P. M., Kramer, D. H., Ultrasound cardiography: contrast studies in anatomy and function. *Radiology* **1969**, *92(5)*, 939–948.

296 Kremkau, F. W., Gramiak, R., Carstensen, E. L., Shah, P. M., Kramer, D. H., Ultrasonic detection of cavitation at catheter tips. *Am. J. Roentgenol. Radium Ther. Nucl. Med.* **1970**, *110(1)*, 177–183.

297 Mattrey, R. F., Wrigley, R., Steinbach, G. C., Schutt, E. G., Evitts, D. P., Gas emulsions as ultrasound contrast agents. Preliminary results in rabbits and dogs. *Invest. Radiol.* **1994**, 29 Suppl. 2, S139–S141.

298 El-Sherif, D. M., Wheatley, M. A., Development of a novel method for synthesis of a polymeric ultrasound contrast agent. *J. Biomed. Mater. Res. A* **2003**, *66(2)*, 347–355.

299 Forsberg, F., Lathia, J. D., Merton, D. A., Liu, J. B., Le, N. T., Goldberg, B. B., Wheatley, M. A., Effect of shell type on the *in vivo* backscatter from polymer-encapsulated microbubbles. *Ultrasound Med. Biol.* **2004**, *30(10)*, 1281–1287.

300 Unger, E. C., McCreery, T., Sweitzer, R., Vielhauer, G., Wu, G., Shen, D., Yellowhair, D., MRX 501: a novel ultrasound contrast agent with therapeutic properties. *Acad. Radiol.* **1998**, 5 Suppl. 1, S247–S249.

301 Unger, E. C., Hersh, E., Vannan, M., Matsunaga, T. O., McCreery, T., Local drug and gene delivery through microbubbles. *Prog. Cardiovasc. Dis.* **2001**, *44(1)*, 45–54.

302 Klibanov, A. L., Targeted delivery of gas-filled microspheres, contrast agents for ultrasound imaging. *Adv. Drug. Deliv. Rev.* **1999**, *37(1–3)*, 139–157.

303 Unger, E. C., Matsunaga, T. O., McCreery, T., Schumann, P., Sweitzer, R., Quigley, R., Therapeutic applications of microbubbles. *Eur. J. Radiol.* **2002**, *42(2)*, 160–168.

304 Chen, S., Shohet, R. V., Bekeredjian, R., Frenkel, P., Grayburn, P. A., Optimization of ultrasound parameters for cardiac gene delivery of adenoviral or plasmid deoxyribonucleic acid by ultrasound-targeted microbubble destruction. *J. Am. Coll. Cardiol.* **2003**, *42(2)*, 301–308.

305 Skyba, D. M., Price, R. J., Linka, A. Z., Skalak, T. C., Kaul, S., Direct *in vivo* visualization of intravascular destruction of microbubbles by ultrasound and its local effects on tissue. *Circulation* **1998**, *98(4)*, 290–293.

306 Price, R. J., Skyba, D. M., Kaul, S., Skalak, T. C., Delivery of colloidal particles and red blood cells to tissue through microvessel ruptures created by targeted microbubble destruction with ultrasound. *Circulation* **1998**, *98(13)*, 1264–1267.

307 Hynynen, K., McDannold, N., Vykhodtseva, N., Jolesz, F. A., Noninvasive MR imaging-guided focal opening of the blood-brain barrier in rabbits. *Radiology* **2001**, *220(3)*, 640–646.

308 Tata, D. B., Dunn, F., Tindall, D. J., Selective clinical ultrasound signals mediate differential gene transfer and expression in two human prostate

cancer cell lines: LnCap and PC-3. *Biochem. Biophys. Res. Commun.* **1997**, *234(1)*, 64–67.

309 Unger, E. C., McCreery, T. P., Sweitzer, R. H., Ultrasound enhances gene expression of liposomal transfection. *Invest. Radiol.* **1997**, *32(12)*, 723–727.

310 Schratzberger, P., Krainin, J. G., Schratzberger, G., Silver, M., Ma, H., Kearney, M., Zuk, R. F., Brisken, A. F., Losordo, D. W., Isner, J. M., Transcutaneous ultrasound augments naked DNA transfection of skeletal muscle. *Mol. Ther.* **2002**, *6(5)*, 576–583.

311 Anwer, K., Kao, G., Proctor, B., Anscombe, I., Florack, V., Earls, R., Wilson, E., McCreery, T., Unger, E., Rolland, A., Sullivan, S. M., Ultrasound enhancement of cationic lipid-mediated gene transfer to primary tumors following systemic administration. *Gene Ther.* **2000**, *7(21)*, 1833–1839.

312 Teupe, C., Richter, S., Fisslthaler, B., Randriamboavonjy, V., Ihling, C., Fleming, I., Busse, R., Zeiher, A. M., Dimmeler, S., Vascular gene transfer of phosphomimetic endothelial nitric oxide synthase (S1177D) using ultrasound-enhanced destruction of plasmid-loaded microbubbles improves vasoreactivity. *Circulation* **2002**, *105(9)*, 1104–1109.

313 Vannan, M., McCreery, T., Li, P., Han, Z., Unger, E., Kuersten, B., Nabel, E., Rajagopalan, S., Ultrasound-mediated transfection of canine myocardium by intravenous administration of cationic microbubble-linked plasmid DNA. *J. Am. Soc. Echocardiogr.* **2002**, *15(3)*, 214–218.

314 Lawrie, A., Brisken, A. F., Francis, S. E., Cumberland, D. C., Crossman, D. C., Newman, C. M., Microbubble-enhanced ultrasound for vascular gene delivery. *Gene Ther.* **2000**, *7(23)*, 2023–2027.

315 Frenkel, P. A., Chen, S., Thai, T., Shohet, R. V., Grayburn, P. A., DNA-loaded albumin microbubbles enhance ultrasound-mediated transfection *in vitro*. *Ultrasound Med. Biol.* **2002**, *28(6)*, 817–822.

316 Shohet, R. V., Chen, S., Zhou, Y. T., Wang, Z., Meidell, R. S., Unger, R. H., Grayburn, P. A., Echocardiographic destruction of albumin microbubbles directs gene delivery to the myocardium. *Circulation* **2000**, *101(22)*, 2554–2556.

317 Song, J., Tata, D., Li, L., Taylor, J., Bao, S., Miller, D. L., Combined shock-wave and immunogene therapy of mouse melanoma and renal carcinoma tumors. *Ultrasound Med. Biol.* **2002**, *28(7)*, 957–964.

318 Miller, D. L., Pislaru, S. V., Greenleaf, J. E., Sonoporation: mechanical DNA delivery by ultrasonic cavitation. *Somat. Cell Mol. Genet.* **2002**, *27(1–6)*, 115–134.

319 Danialou, G., Comtois, A. S., Dudley, R. W., Nalbantoglu, J., Gilbert, R., Karpati, G., Jones, D. H., Petrof, B. J., Ultrasound increases plasmid-mediated gene transfer to dystrophic muscles without collateral damage. *Mol. Ther.* **2002**, *6(5)*, 687–693.

320 Lu, Q. L., Liang, H. D., Partridge, T., Blomley, M. J., Microbubble ultrasound improves the efficiency of gene transduction in skeletal muscle *in vivo* with reduced tissue damage. *Gene Ther.* **2003**, *10(5)*, 396–405.

321 Endoh, M., Koibuchi, N., Sato, M., Morishita, R., Kanzaki, T., Murata, Y., Kaneda, Y., Fetal gene transfer by intrauterine injection with microbubble-enhanced ultrasound. *Mol. Ther.* **2002**, *5(5 Pt 1)*, 501–508.

322 Fischer, A., Hacein-Bey, S., Cavazzana-Calvo, M., Gene therapy of severe combined immunodeficiencies. *Nat. Rev. Immunol.* **2002**, *2(8)*, 615–621.

323 Hacein-Bey-Abina, S., von Kalle, C., Schmidt, M., Le Deist, F., Wulffraat, N., McIntyre, E., Radford, I., Villeval, J. L., Fraser, C. C., Cavazzana-Calvo, M., Fischer A. A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N. Engl. J. Med.* **2003**, *348(3)*, 255–256.

324 Somia, N., Verma, I. M., Gene therapy: trials and tribulations. *Nat. Rev. Genet.* **2000**, *1(2)*, 91–99.

6
DNA Needle Injection

Matthias Giese

6.1
From Mouse to Human

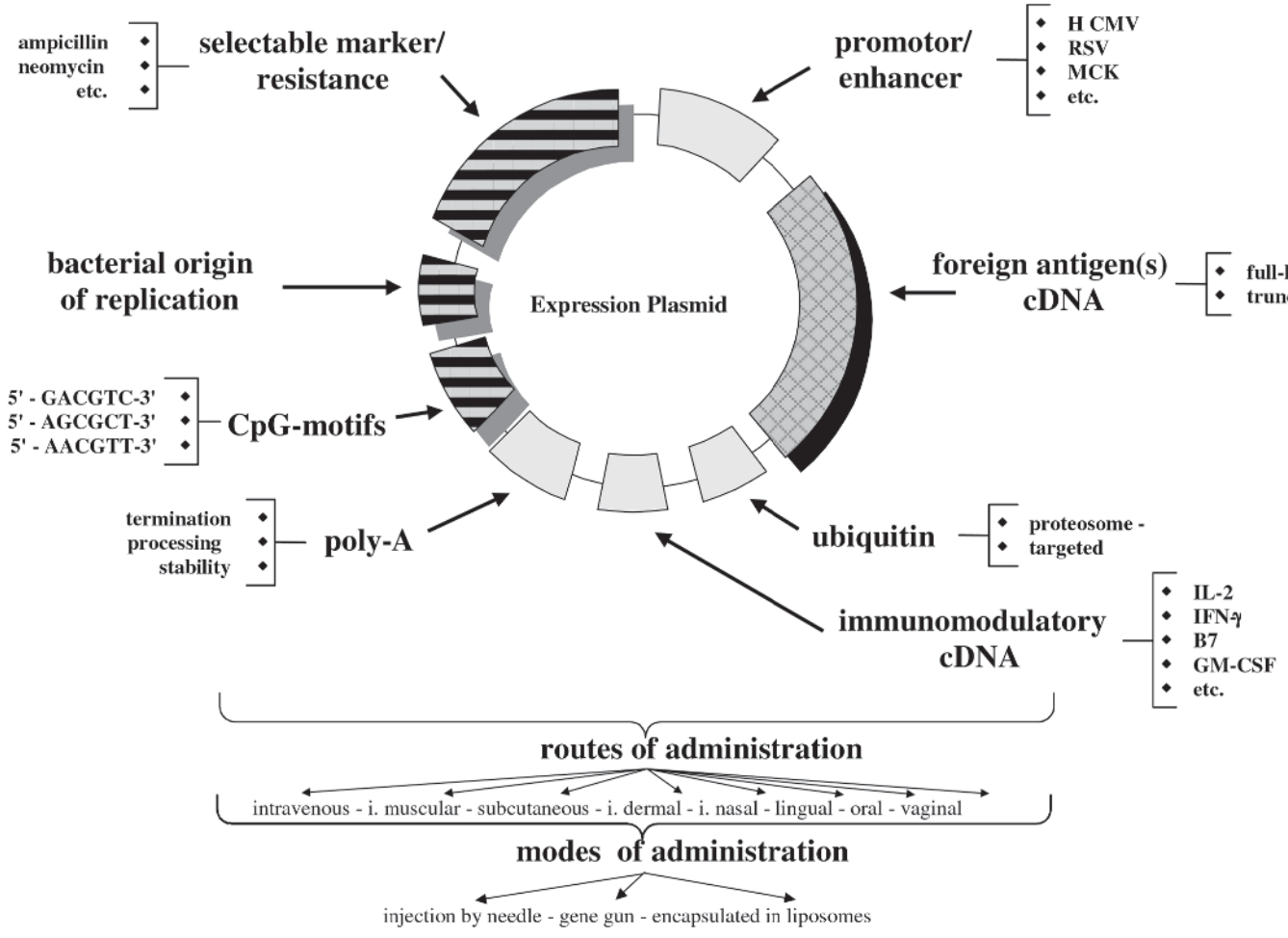
There is still an unmet need for effective vaccines against various diseases. The reason is a lack of safe and effective vaccine against some important infections and other infectious diseases that to this day can still be fought only insufficiently with classical vaccines. The focus of modern vaccine developments, however, is also now on noninfectious diseases, with genetic engineering opening up new possibilities.

The need for new, biologically safe, and immunologically effective vaccines is therefore apparent.

Within the last 12 years a new vaccinating principle, activation of the immune system by means of DNA vaccination, has been intensively investigated and developed. DNA vaccination involves the application of pure plasmid DNA incorporated in an eukaryotic expression vector geared to activate both arms of the immune system: the humoral and the cellular. Although the general application route is by intramuscular injection, various other routes – subcutaneous, intravenous, intranasal, and oral – are also effective [1].

6.1.1
DNA Vaccines

The special quality of DNA vaccines is that they partly imitate the natural infection of a virus – adsorption, penetration, and final budding at the cell membrane – without being pathogenous. After application, the plasmid DNA penetrates into the cell, finds its way through the cytoplasm to the nucleus of the cell, is activated and transcribed like any other (genomic) DNA, and is finally transported as mRNA back into the cytoplasm for translation into protein. The *antigens* thus generated are presented to the immune system, which they activate. Figure 6.1 schematically illustrates a typical expression plasmid as used for the DNA vaccine.


ampicillin
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foreign antigen(s)
cDNA
full-length
truncated
5' - GACGTC-3'
5' - AGCGCT-3'
5' - AACGTT-3'
CpG-motifs
termination
processing
stability
poly-A
ubiquitin
proteosome -
targeted
immunomodulatory
cDNA
IL-2
IFN-γ
B7
GM-CSF
etc.
routes of administration
intravenous - i. muscular - subcutaneous - i. dermal - i. nasal - lingual - oral - vaginal
modes of administration
injection by needle - gene gun - encapsulated in liposomes

6.1.2 Successful Strategy for Vaccination

A successful vaccinating strategy must meet at least four fundamental criteria:

- The vaccine must produce a broad protective immunity in a high percentage of vaccinees.
- The vaccine must induce a broad and longlasting immunological memory.
- The vaccine must be biologically safe and tolerable in a high percentage of vaccinees.
- The vaccine must be produced according to international standards, according to GMP guidelines.

DNA vaccines can be experimentally applied by different routes:

• into the muscle:	by injection by gene gun (Chapter 10)
• into the skin:	by injection (Chapter 7) by gene gun (Chapter 10)
• into the blood system:	by infusion/injection (Chapter 9)
• into the respiratory tract:	intranasal (e.g. spray) (Chapters 5 and 8) intratracheal (e.g. spray) (Chapters 5 and 8)
• into the digestion system:	oral (capsule)
• into the urogenital tract:	by gene gun (Chapter 10) by instillation

Of the various sorts of experimental approaches with laboratory animals, intramuscular (i.m.) immunization by needle and syringe has gained acceptance as a successful application method both for large animals and for studies in humans.

Since the classical intradermal (i.d.) route is increasingly being replaced by needleless injection, only a short description is given here (in Section 6.2, on the special immunology of the skin and its great importance for DNA vaccination by needle).

Needleless injection is reviewed in detail in Chapter 7 of this book.

Figure 6.1 Schematic diagram of an expression plasmid used for DNA vaccination. Individual elements comprising functional expression cassettes. The encoded antigen, as full-length or truncated cDNA, is under the control of strong promotor/enhancer and polyadenylation sequences. Coexpression of cytokines will specifically enhance the immune response. Unspecific activation of the immune system can be provoked by CpG islands. These CpG motifs are part of the bacterial backbone of the plasmid (black strips on the left). Vaccines that focus only on a strong CTL response can be enhanced by coexpression of ubiquitin to target the proteosome pathway. After purification, plasmid DNA is reconstituted in sterile saline or attached to gold particles and can be used for vaccination (M. Giese, 1998).

6.2 Intramuscular Injection

One benefit of i.m. injection is the great volume that can be applied: up to 10 mL. DNA vaccines, however, should be injected with average volumes of only 1–2 mL for large animals and humans. Some muscle fibers will be destroyed during this procedure, though, and it is accompanied with a reversible healing. The regeneration time can last up to a year. Re-vaccinations therefore should not be carried out in the "old" vaccinating site, since the effect of the vaccination could be unsuccessful because of the destroyed fibers.

(Skeletal) muscle fibers are unusual and unique in various ways, not only in terms of anatomy. These fibers are full of actin and myosin elements, and are driven by large energy supplies. Their mode of regeneration is also different from other body cells; a single muscle fiber cell represents a giant protein biosynthesis factory and skeletal muscle cells are longlasting, most of them probably living as long as the animal.

6.2.1 Biology of Muscle Fibers

On intramuscular application into the arm or leg the DNA vaccine hits skeletal muscle.

The skeletal muscle is responsible for all voluntary movements and is one of four different mammalian muscle cell types, together with the cardiac muscle, the myoepithelian cells, and the smooth muscle.

Skeletal muscle consists of muscle cells, which are described as muscle fibers because of their form and are wrapped by connective tissue on the outside.

Muscle fibers (= muscle cells) are geared towards doing mechanical work, primarily by contracting themselves. Over two thirds of a muscle fiber is made up of myofibrils, mostly long, spindle-shaped entities that are able to contract themselves thanks to their myosin/actin elements. A skeletal muscle fiber therefore represents one, exceptionally large, single cell. One such large human muscle cell can reach up to half a meter long, with a diameter of up to 100 µm. These giant cells have arisen from the fusion of many single skeletal muscle cells. Every muscle cell is therefore a syncytium with many nuclei, which lie on the edge of the muscle cell, so a skeletal muscle cell is multinucleate [2]. These multinuclei and the surrounding great cytoplasm are unique in the body.

6.2.1.1 Resting Stem Cells

All nuclei in a muscle cell contain diploid DNA, but they are not able to replicate this DNA: skeletal muscle cells cannot divide themselves. Cells lost by injury or for some other reason are not replaced by mitosis of the intact adult muscle cells, but replacement is instead achieved by "embryonic" myoblasts, which reside as so-called satellite cells in the skeletal muscle fibers and become active to form the basis for the repair as required. The myoblasts are "selfmade" cells, renewable at

any time, and constitute the basis for all differentiated skeletal muscle cells. The myoblasts are the stem cells of the skeletal muscle.

6.2.2 Uptake of Plasmid DNA

Plasmid DNA is probably actively taken up by cell membrane receptors and internalized into the cytoplasm [3]. In the nucleus this plasmid DNA cannot replicate or integrate, so a high degree of biological safety is offered [4–8].

What influence the multinuclei have on the expression rate of a DNA vaccine has yet to be examined and so may only be speculated upon. Unlike in a mononuclear cell, though, the plasmid DNA has to overcome the "cell membrane" barrier only once to reach the cytoplasm.

Muscle cell cytoplasm itself represents a large cytoplasmatic unity with multiple nuclei. Consequently, the ribosomes are multiplied too, so plasmid DNA in this giant muscle cell encounters a highly potent protein factory. While the contractions of a muscle cell occur synchronously, nuclei activities and protein biosynthesis are not synchronized in this multinuclei and multiribosomal cell.

What does this all mean for a DNA vaccine?

The plasmid DNA enters into a large, unitary cytoplasm and can now choose between many nuclei for transcription. These nuclei differ in their activities, so that the mRNA may become translated into protein either all in parallel or spread over some period of time.

On the one hand, massive production of these antigens might be achievable by this solid *ad hoc* translation, on the other hand the antigens might be produced continuously over a longer time period. This would also explain the depository effect of muscle cells for vaccination with DNA. Another advantage of these cellular myonuclei complexes lies in the fact that possible faulty gene copies produced in nucleus A or B can be compensated for by other, correctly working nuclei C, D, E, etc.

The plasmid DNA is so efficiently transcribed in the nucleus, and translated into protein in the cytoplasm, that these new antigens are able to activate the immune system completely.

And this activation process is exactly the problem of the immunization of muscle cells, the "*Achilles heel*": Muscle cells cannot activate the immune system *per se*.

6.2.3 Activation of the Immune System

Are muscle cells suitable for DNA vaccination at all?

From the point of view of protein biosynthesis this could be the case. Successful immunization, however, requires specific activation of the complete immune system with antibodies and CTLs, and different mechanisms are necessary. B cells recognize

soluble antigen, which freely swims in the cytosol; T cells, however, recognize only receptor-bound antigen.

The central issue of muscle immunization with DNA is, therefore, are muscle cells able to process antigens and also to present them?

To answer this question, we should take a look at the main cells involved in the specific immune answer.

The most important cells are particularly the B and T lymphocytes, characterized by membrane-bound receptor molecules through which antigens are recognized and bound on the surface. Beside these B and T cells, a third type cell is necessary, and this has the most important job in this context: the capture of antigen and the presentation of antigen to T cells. Without any presentation, activation of T cells will be unsuccessful. (Textbooks on immunology are recommended here for deeper discussion of immune reactions.)

6.2.3.1 **Receptors and other Signals**

The receptors of B and T cells are quite different.

B cells produce antibodies and use membrane-bound antibodies as receptors to bind a soluble antigen, so B cells use immune globulins.

T cells, however, do not recognize any free antigen. They are instructed with the help of "professional" antigen-presenting cells (APCs), which carry molecules of the major histocompatibility complex, MHC class I and class II. Complexed with those MHC molecules on the surface, the antigen is presented to the T cells.

T_H cells, helper cells, are characterized by CD4+ structures and bind APC MHC class II molecules. T_{H1} or T_{H2} cells produce, among other compounds, various cytokines, such as interleukins (IL2, IL4, IL5) and interferons, such as IFN gamma etc. Effector T cells, T_E cells, characterized by CD8+ structures, bind APC MHC class I molecules. These T cells are cytotoxic.

APCs possess both MHC class I and class II molecules. They are present in the skin as Langerhans cells and as dendritic cells (DCs) in the secondary lymphoid organs and in the thymus. All APCs present antigen on their cell surfaces. The CD4+ cells support both CD8+ T cells and B cells through their cytokines.

The APCs still have additional, costimulatory signals, such as ICAM 1, CD11b, or CD80, so that T cells will be sufficiently activated. APCs activate both T_H and T_E cells; that is, CD4+ and CD8+ cells.

Such high densities of MHC molecules and costimulatory signals as are present on APCs cannot be found in any other body cell. Without these costimulatory signals no effective T cell activation is possible. Antigen presentation without these signals causes T cell tolerance and would make a vaccine ineffective [10].

6.2.3.2 **Antigen Presentation**

Effector T cells have the ability to migrate out of lymphoid tissue into the non-lymphoid tissue, to the sites of virus replication. This migration is regulated through adhesion molecules, such as integrins, selectins, and homing and chemokine receptors on T cells.

Table 6.1 Tools for antigen presentation and characteristics of "professional" antigen-presenting cells (APCs) compared with skeletal muscle cells.

Cell equipment	*Cell type*	
	APC	*Skeletal muscle cell*
Antigen capture	yes	no
Antigen processing	yes	no
Antigen presentation	yes	no
MHC class I	yes	yes
MHC class II	yes	no
Co-stimulatory signal	yes	no
Chemokines	yes	no
Migration	yes	no

APCs produce molecules that attract T cells to the site of the event, and also have the capability for migration, so both APCs and T cell types are ideal partners [11].

The antigen is captured by APCs by pinocytosis and is processed into immunogenic fragments. This antigen processing is followed by antigen presentation on the surface.

A large number of conditions must be filled for successful presentation of any antigen to T cells. Last but not least, the strength of the binding capacity between the *APC–MHC-antigen complex* and the *T cell receptor* (TCR) also strongly influences the T cell activation.

Table 6.1 compares the characteristics of APCs with those of skeletal muscle cells.

6.2.4 Cross-Priming

Obviously the skeletal muscle does not have any special molecular tools to stimulate the immune system effectively, except for the ubiquitous MHC I receptors.

Nevertheless, vaccination directly into muscle shows success. Many studies from laboratory animals to large animals to human clinical studies have shown that i.m. DNA vaccination is able to activate the immune system completely.

Wolff first reported direct injection of (plasmid) DNA into skeletal muscle in 1990 [12]. Longlasting immune responses are obtained in many cases without boost [13, 14]. This has been thought to be due to the fact that mature muscle fibers are postmitotic, so expression of the episomally located plasmid DNA can continue for prolonged periods of time.

Indeed, it has been shown that expression of luciferase gene injected directly into mouse muscle can still be detected 19 months later [15]. In a mouse model,

Yokoyama et al. [16a] were able to demonstrate an impressive difference in immunity depending both on the muscle injected and on the dose of DNA administered [16]. They used three criteria – CTL (cytotoxic T lymphocyte) induction, reduction of virus titer, and survival rate following challenge with a lethal dose of virus – and found that immunity induced by DNA injection of the anterior tibial muscle significantly exceeds that induced after injection of the quadriceps muscle.

The same group also reported that intradermal DNA application with a needle could induce a stronger immune response than intramuscular inoculation with the same amount of DNA (see also Section 6.3, intradermal injection).

6.2.5 Safety Aspects

Of special interest regarding the safety of a DNA vaccine is the question of whether the injected DNA is able to induce the generation of DNA autoantibodies, to induce or to accelerate autoimmunity.

Answers to this question were supplied by Mor et al. [17], who repeatedly intramuscularly immunized Balb/c mice variously with plasmid DNA encoding the malaria CS 1 protein or the HIV gp 160 protein, or with plasmid devoid of insert. A threefold increase in the number of B cells secreting IgG antibodies against mammalian double-stranded DNA was measured in normal mice, but none of the plasmids used in these experiments elicited antimuscle cell autoantibodies. Long-term studies of normal and lupus-prone mice showed that repeated administration of DNA vaccines did not induce or accelerate myositis or systemic autoimmune disease. These findings suggest that DNA vaccines neither initiate nor accelerate the development of systemic autoimmunity.

Three main processes are decisive for immune activation after intramuscular application:

- uptake of the DNA by muscle cells,
- antigen processing,
- antigen presentation.

6.2.5.1 Uptake of the DNA by Muscle Cells

Uptake is independent of the cell type, receptor-mediated, and energy-dependent, so this process takes place in the muscle cell just like in other cells.

6.2.5.2 Antigen Processing

No specific antigen processing takes place in the muscle cell. General protein biosynthesis is independent of the DNA vaccination however, so the plasmid DNA is translated into protein like other genes. The vaccinated muscle cell cannot use these foreign proteins, however, and will release them to the normal turnover of (such) proteins: labeled by ubiquitines and final degradation.

6.2.5.3 **Antigen Presentation**

The release of the vaccinated antigens by the muscle cell is therefore a decisive prerequisite for the activation of the immune system. How does this happen?

The antigen could be delivered by secretion or by dying, apoptotic muscle cells. Apoptotic cell death could be induced spontaneously (by the use of needles, for example), by hydrostatic pressure on injection with saline, or, consistently with general rules of immunology, by CTL activity against "infected" muscle cells presenting foreign peptides complexed with class I molecules at the surface [18–20].

It should be noted that in some experimental attempts have been made to increase protein expression by pretreatment with agents that cause muscle fiber destruction and ensuing muscle regeneration, such as bupivacaine [21, 22] and cardiotoxin [23], or pretreatment with hypertonic sucrose. These changes, ups and downs, cause the death of muscle cells, followed by recruitment of immune cells to the site of tissue damage.

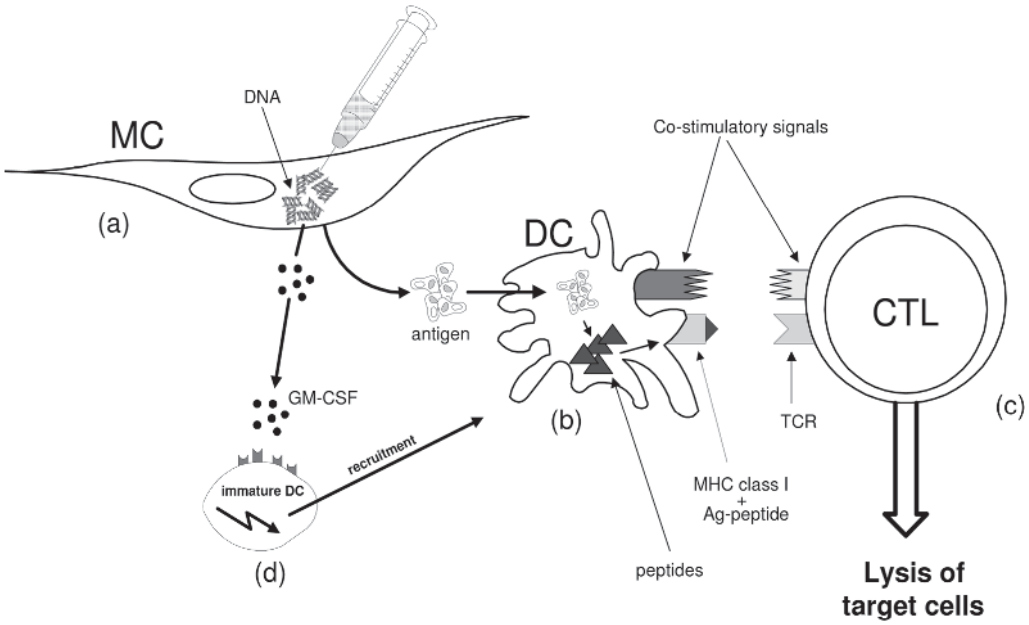

Figure 6.2 Cross-priming is an alternative mechanism by which "professional" bone marrow-derived antigen-presenting cells (APCs) may process exogenous antigens for presentation to cytotoxic T lymphocytes (CTLs) *in vivo*. Class I – restricted presentation is usually associated with cytoplasmic degradation of cellular proteins and is often considered inaccessible to exogenous antigens. Cross-priming, however, can circumvent this classical pathway by indirect presentation. (a) Muscle cells (MCs) are vaccinated with expression plasmid DNA. (b) Antigens from MCs are acquired by dendritic cells (DCs) and Ag-peptides are presented on MHC class I molecules to specific T cell receptors (TCRs) in combination with costimulatory signals to precursor CTLs. (c) Primed CTLs are now able to attack specific target cells. (d) Granulocyte-macrophage colony stimulating factor (GM-CSF), as part of a DNA vaccine, can enhance the immune response and recruit DCs to the site of action. (M. Giese, 1998)

Muscle cells cannot drive clonal expansion of T cell, the production of cytokines, and development into killer cells because they lack appropriate costimulatory signals that are expressed by APCs and only weakly express MHC class I molecules.

A "mediator cell" between DNA-transfected muscle cells and T cells is necessary. Indeed, Corr [24] was able to demonstrate that muscle cells do not themselves present gene-encoded proteins to the immune system. This presentation occurs at the surface of a "mediator cell": a "professional" bone marrow-derived APC, especially a DC responsible for priming T cells.

DCs can take up particles and microbes, but also cell debris, by phagocytosis. DCs will take up secreted antigens via pinocytic vesicles, in which extracellular fluids and solutes are sampled.

An exogenous (*scavenger*) pathway will present such "external" antigens – not processed by the classical pathway through proteosomes, cytosol, and endoplasmatic reticulum, where they bind to class I molecules.

The scavenger pathway is also necessary for antigens derived from tumor cells or transplants, or antigens from viruses that cannot infect DCs.

This kind of processing of external antigens is called cross-priming and is illustrated in Figure 6.2. In contrast, antigens synthesized in the cytoplasm of DCs or other APCs would clearly have direct access to class I MHC processing classical pathways.

6.2.6
DNA Vaccination of Horses against Infection with Equine Arteritis Virus I

We have developed various DNA vaccines against the infection of horses with the equine arteritis virus (EAV). EAV is a small, single-stranded RNA virus (12 kb) belonging to the arteriviridae family, as described in [25].

A schematic diagram of the virus is given in Figure 6.3.

When we started our experiments nothing was known about the possible immunogenicity of ORF2. We immunized mice with ORF2 (basic immunization 1000 µg i.m., followed by two boosters at four-week intervals with the same DNA amount i.m., but were not able to induce any strong immune response. ORF2, or its corresponding gene product, seemed to be not immunogenic enough.

The mice were not the natural host of EAV, though, so we changed to horses. Two horses were immunized with a DNA vaccine expressing the minor glycoprotein of equine arteritis virus encoded by EAV-ORF2. In addition, this vaccine also contained an expression plasmid for equine interleukin 2 to stimulate the cellular immune response. The results of the NT-tests are summarized in Table 6.2.

Both horses responded to the ORF2 antigen [26], the first antibodies being detectable four weeks after the basic immunization. Muscle cells seem to act as depots for the injected DNA and so will influence the duration and the stability of immunity. The longlasting immune response after i.m. injection could be due to the long-term expression of the target antigen by muscle cells as discussed above.

About 12 weeks after the basic immunization a stabilized mean titer of neutralizing antibodies was measurable, and this humoral immunity was stable over eight

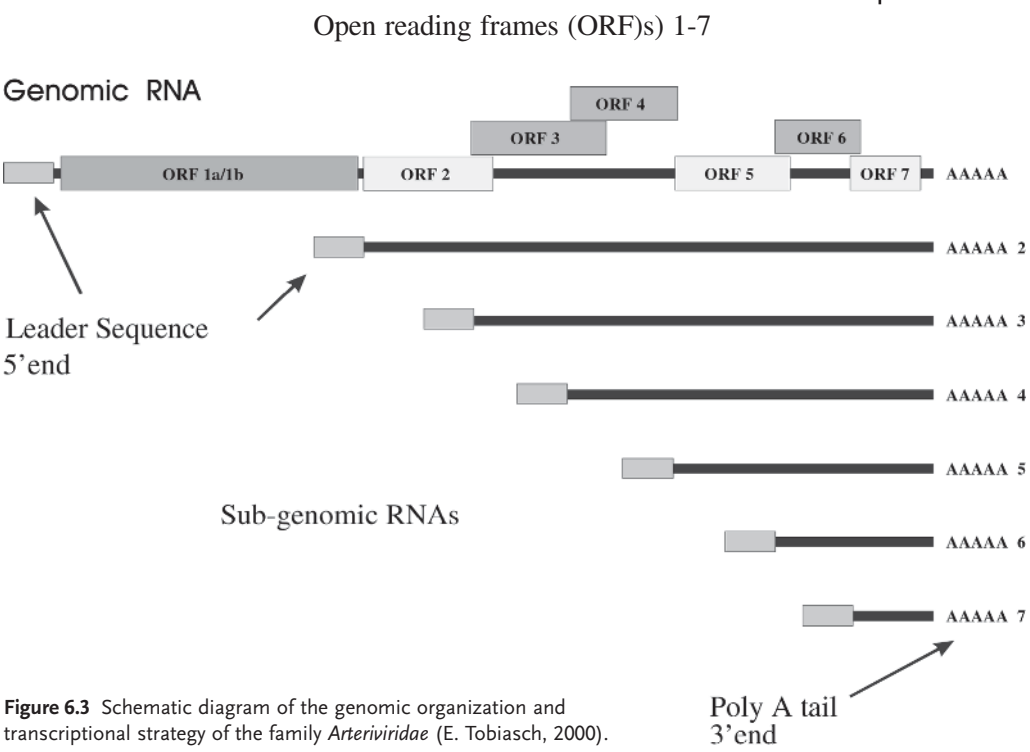

Figure 6.3 Schematic diagram of the genomic organization and transcriptional strategy of the family *Arteriviridae* (E. Tobiasch, 2000).

Table 6.2 Results of neutralization tests (NTs) against equine arteritis virus (EAV) after DNA vaccination with cDNA of EAV-ORF2 and equine interleukin 2 (eqIL-2). Control serum (prior to vaccination), post vaccination serum (no. of weeks after vaccination).

Time points of NT tests	***NT titer***	
	Horse A	***Horse B***
Prevaccination control	< 1 : 2	< 1 : 2
4 weeks post vaccination	< 1 : 2	< 1 : 2
8 weeks post vaccination	1 : 32	1 : 128
12 weeks post vaccination	1 : 128	1 : 128
18 weeks post vaccination	1 : 128	1 : 128
21 weeks post vaccination	1 : 128	1 : 128
25 weeks post vaccination	1 : 96	1 : 64
30 weeks post vaccination	1 : 32	1 : 128
34 weeks post vaccination	1 : 96	1 : 128

months. These results confirm our previous studies [25] that an EAV-DNA vaccination induces a stable and longlasting immune response in horses.

A special focus of this study has been the induction of the cellular immune response. Both horses developed a CTL activity after immunization with EAV ORF2, and this cellular immune response was measurable over three months of observation.

6.3 Intradermal Injection

Intradermal, synonym: intracutaneous (or intradermic), relating to areas between the layers of the skin.

The skin is the largest human organ, its surface area being about 1.6 m^2 and its weight almost a sixth of total body weight. The most fundamental difference between i.d and i.m. vaccination are the tissue cells involved.

For better understanding of the biology associated with DNA vaccination, we should take a look at the anatomy of the skin.

There are different skin layers from the top to the bottom:

_____outside_____

- epidermis — keratinocytes, melanocytes, T cells, Langerhans cells
- corium — fibroblasts, T cells, macrophages, mastcells, Langerhans cells
- (sub)cutis — foam cells

_____inside_____

6.3.1 Skin-Associated Lymphoid Tissue (SALT)

On the way to the corium the intradermal injection penetrates the epidermis, which houses keratinocytes and melanocytes, together with T cells and resting Langerhans cells. The injection then hits the corium, which accommodates fibroblasts, lymphocytes and macrophages, mast cells, and again Langerhans cells.

With such a repertoire of immunocompetent cells, the skin is an ideal site for DNA vaccination. The plasmid DNA hits an environment of highly concentrated APCs, effector and regulator cells.

Macrophages have some of the highest secretion rates of all body cells, with more than 100 different products. They are also the most prominent phagocytotic cells and are closely engaged in immune regulation through antigen processing and presentation to lymphocytes.

Typical products of macrophages are IL-1, IL-6, IL-8, and IL-10, together with IFN alpha/beta and tumor necrosis factor (TNF) alpha.

IFN alpha/beta has a special function in this complex, upregulating MHC class I molecules and supporting antigen processing and presentation.

Macrophages express both classes of MHC molecules on their surfaces, although only 15% of all macrophages are estimated to express MHC class II molecules. Macrophages are not the best cells for antigen presentation, but a small group of them are able to do it.

Langerhans cells in the skin are extremely useful. These cells are a subgroup of dendritic cells and therefore "professional" antigen-presenting cells.

Taken together, the skin is an essential part of the immune system, with antigen-presenting DCs, circulating T lymphocytes (but no B cells), immunoregulatory macrophages, and keratinocytes producing cytokines. This immunological skin network is described as skin-associated lymphoid tissue, SALT [27, 28].

The great benefit for DNA vaccination by the intradermal route is the direct access to "professional" APCs. Once antigens are taken up by stimulated APCs in the skin, the APCs migrate to regional draining lymph nodes in order to activate T cells.

This direct activation of T cells is of course faster than the indirect way through muscle cell vaccination. We have been able to demonstrate this with horses immunized by i.d application and by i.m. application as described. The muscle cells, however, build up a powerful depot of the antigen and influence the duration of immune response.

Both advantages – fast immune responses and longlasting ones – are very useful, and both application routes are therefore often combined in one DNA vaccination cycle. Whereas needle and syringe are still used for i.m. injections, needleless injection devices are increasingly replacing the needle for i.d. application. One reason is the relatively difficulty of intradermal needle injection, which needs special training.

It is important to note here that the only successful routes for DNA vaccination in all species investigated, from mouse to human, are intradermal and intramuscular application either by needle or by needleless device.

6.3.2
DNA Vaccination of Horses Against Infection with Equine Arteritis Virus II

In a second experiment we immunized horses with DNA, injected both into to the muscle by needle and intradermally by gene gun ([25] and Chapter 10).

Four vaccinations per animal were given: a basic immunization and three boosters at intervals of about 14 days. The first immunization was on day 0, the second immunization on day 14, the third on day 29, and the fourth on day 51.

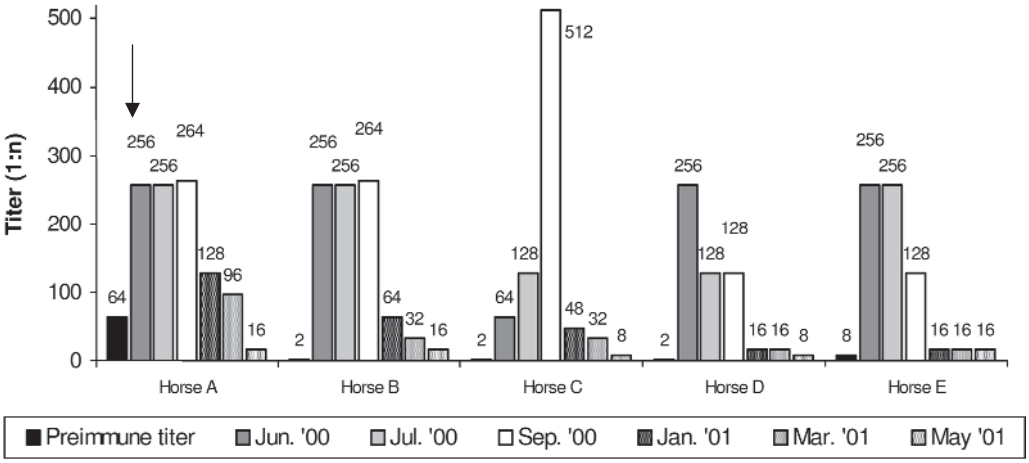

Figure 6.4 Duration of immunity. Neutralizing antibody titers in sera of vaccinated horses (EAV ORF2, 5, 7) were determined. Preimmune sera were measured one day prior to immunization. The vertical arrow, representatively shown for horse A only, denote times of vaccination (basic immunization and three boosters at two-week intervals). The columns represent the SNT titer for different time points over a period of 12 months: SNT-titers for June–July–September 2000 and January–March–May 2001 (M. Giese, 2002).

Each vaccination represents a combination of gene gun and i.m. injection:

Gene Gun: the DNA content of one individual expression plasmid for gene gun was 0.5 µg per shot, or in total 3.5 µg per shot. Each cartridge represented a single shot and contained 3.5 µg DNA, corresponding to 0.5 µg of individual expression vectors. Ten shots on different shaved sites were given, or 35µg per vaccination per animal in total.

The humoral immune responses of the vaccinated horses are illustrated in Figure 6.4.

We measured a very rapid onset of antibody production against the antigens of the vaccine ORFs, with four of the five horses having already developed high titers of neutralizing antibodies after two weeks, as summarized. This is independent of the preimmune status of the horses, and also independent of the race. We measured antibodies against each individual gene product, indicating that the naive DNA of recombinant plasmids harboring ORF2, ORF5, and ORF7 is able to express the corresponding gene products (small viral glycoprotein, major glycoprotein, and nucleocapsid protein; data not shown).

Another important aspect is the duration of immunity. The basic vaccination started in May 2000 and the last serum sample for the SNT check was taken in May 2001. We monitored the development of the immune response over a year by measuring the neutralizing antibodies. Figure 6.4 illustrates these results. There is a plateau of immune response over four to five months after vaccination. All horses showed this plateau with an individual titer. The decline of the titer begins after six to seven months and is measurable in all vaccinated animals, but all horses

still have a protective antibody titer after 12 months. We assume that the described DNA vaccine with ORF2, ORF5, and ORF7 is able to provoke a longlasting humoral immune response.

6.4 Concluding Remarks

Needleless application devices for DNA vaccines are increasingly replacing the classical needle and syringe, especially for the intradermal route. Many studies have demonstrated that needleless injection is safe and able to produce a larger distribution pattern of the plasmid DNA than needle injection [29]. Antibody response is also enhanced by needleless injection, by up to 50-fold compared to the classical needle application [30], with only a fraction of DNA typically used for needle injection.

Nevertheless, this old application method is safe, simple, efficacious and very cheap for intramuscular injection, so needle and syringe seem set to survive in the immediate future.

References

1 Giese, M., *Virus Genes* **1998**, *17*, 219–232.
2 Kumar, A., Velloso, C. P., Imokawa, Y., Brockes, J. P., *PLOS Biology* **2004**, *2*, 1168–1176.
3 Moelling, K., *Cytokines, Cell. and Mol. Therapy.* **1997**, *3*, 127–136.
4 Epstein, J. E., Charoenvit, Y., Kester, K. E., Wang, R., Newcomer, R., Fitzpatrick, S., Richie, T. L., Tornieporth, N., Heppner, D. G., Ockenhouse, C., Majam, V., Holland, C., Abot, E., Ganeshan, H., Berzins, M., Jones, T., Freydberg, C. N., Ng, J., Norman, J., Carucci, D. J., Cohen, J., Hoffman, S. L., *Vaccine* **2004**, *22*, 1592–1603.
5 Kang, K. K., Choi, S. M., Choi, J. H., Lee, D. S., Kim, C. Y., Ahn, B. O., Kim, B. M., Kim, W. B., *Intervirology* **2003**, *46*, 270–276.
6 Ledwith, B. J., Manam, S., Troilo, P. J., Barnum, A. B., Pauley, C. J., Griffiths, T. G., Harper, L. B., Beare, C. M., Bagdon, W. J., Nichols, W. W., *Intervirology* **2000**, *43*, 258–272.
7 Haworth, R., Pilling, A. M., *Hum. Exp. Toxicol.* **2000**, *19*, 267–276.
8 Nichols, W. W., Ledwith, B. J., Manam, S. V., Troilo, P. J., *Ann. Acad, N. Y., Sci.* **1995**, *772*, 30–39.
9 Leitner, W. W., Ying, H., Restifo, N. P., *Vaccine* **1999**, *18*, 765–777.
10 Boise, L. H., Minn, A. J., Noel, P. J., June, C. H., Accavitti, M. A., Lindsten, T., Thompson, C. B., *Immunity* **1995**, *3*, 87–98.
11 Diana, J., Persat, F., Staquet, M. J., Assossou, O., Ferrandiz, J., Gariazzo, M. J., Peyron, F., Picot, S., Schmitt, D., Vincent, C., *FEMS Immunol. Med. Microbiol.* **2004**, *42*, 321–331.

12 Wolff, J. A., Malone, R. W., Williams, P., Chong, W., Ascadi, G., Jani, A., Felgner, P. L., *Science* **1990**, *247*, 1465–1468.

13 Hartikka, J., Sawdey, M., Cornefert-Jensen, F., Margalith, M., Barnhart, K., Nolasco, M., Vahlsing, H. L., Meek, J., Marquet, M., Hobart, P., Norman, J., Manthorpe, M., *Hum. Gen. Ther.* **1996**, *7*, 1205–1217.

14 Darquet, A. M., Cameron, B., Wils, P., Scherman, D., Cronzet, J., *Gene Ther.* **1997**, *4*, 1341–1349.

15 Wolff, J. A., Ludtke, J. J., Acsadi, G., Williams, P., Jani, A., *Hum. Mol. Genet.* **1992**, *1*, 363–369.

16 Davis, H. L., Mancini, M., Michel, M. L., Whalen, R. G., *Vaccine* **1997**, *15*, 553–560.

16a Yokoyama, M., Hassett, D. E., Zhang, J., Whitton, J. L., *Vaccine* **1996**, *14*, 910–915.

17 Mor, G., Singla, M., Steinberg, A. D., Hoffmann, S. L., Okuda, K., Klinman, D. M., *Hum. Gen. Ther.* **1997**, *8*, 293–300.

18 Payette, P. J., Weeratna, R. D., McCluskie, M. J., Davis, H. L., *Gene Ther.* **2001**, *8*, 1395–1400.

19 Rock, K. L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., Goldberg, A. L., *Cell* **1994**, *78*, 761–771.

20 Coux, O., Tanaka, K., Goldberg, A., *Annu. Rev. Biochem.* **1996**, *65*, 801–847.

21 Lewis, P. J., Cox, G. J. M., van Drunen, S., van den Hurk, L., Babiuk, L. A., *Vaccine* **1997**, *15*, 861–864.

22 Nakano, I., Maertens, G., Major, M. E., Vitvitski, L., Dubuisson, J., Fournillier, A., de Martynoff, G., Trepo, C., Inchauspe, G., *J.Virol.* **1997**, *71*, 7101–7109.

23 Boyle, J. S., Bradly, J. L., Lew, A. M., *Nature* **1998**, *392*, 408–410.

24 Corr, M., Lee, D. J., Carson, D. A., Tighe, H., *J. Exp. Med.* **1996**, *184*, 1555–1560.

25 Giese, M., Bahr, U., Jakob, N. J., Kehm, R., Handermann, M., Muller, H., Vahlenkamp, Th., Spiess, C., Schneider, Th., Schusser, G., Darai, G., *Virus Genes* **2002**, *25*, 159–167.

26 Giese, M., Schneider, Th., Schusser, G., Liebert, U., *J. Gen. Virol.*, submitted **2004**.

27 Streilein, J. W., Alard, P., Niizeki, H., *Keio. J. Med.* **1999**, *48*, 22–27.

28 Streilein, J. W., *Immunol. Ser.* **1989**, *46*, 73–96.

29 Baizer, L., Hayes, J., Lacey, C., D'Antonio, L., *Pharm. Manu. Pack. Resou.* **2002**, Spring, 96–100.

30 Aguiar, J. C., Hedstrom, R. C., Rogers, W. O., Charoenvit, Y., Sacci, J. B., Lanar, D. E., Majam, V. F., Stout, R. R., Hoffman, S. L., *Vaccine* **2001**, *20*, 275–280.

7
Needleless Jet Injection of Naked DNA for Nonviral *in vivo* Gene Transfer

Wolfgang Walther and Ulrike Stein

7.1
Introduction

The transfer of naked DNA for nonviral gene therapy represents an alternative to viral and liposomal gene transfer technologies (see also Chapter 5), with increasing importance for use in genetic immunization (see also Chapter 6), DNA vaccination, gene immune therapy approaches, and other gene therapy applications [1–4]. The attractiveness of the use of naked DNA gene transfer technologies is reflected in the fact that about 15% of all gene therapy trials are currently based on naked DNA gene transfer.

For the delivery of naked DNA into targeted cells or tissues, a great variety of procedures are employed both *in vitro* and *in vivo*. One early described procedure for naked DNA transfer was simple needle and syringe injection, which has now been developed into a hydrodynamics pressure method that applies relatively large volumes within short times ([1, 2] and Chapter 9).

During the last decade various physical methods, including particle bombardment (see Chapter 10), *in vivo* electroporation (see Chapters 11 and 12), and jet injection, have evolved into applicable techniques for *in vitro* and *in vivo* gene transfer [5–10]. The advantage of all these nonviral technologies is the circumvention of the use of recombinant viral particles (such as retroviral or adenoviral vectors), preventing side effects associated with viral gene transfer, including immune response towards viral proteins, virus-induced insertional mutagenesis, or viral recombination. In fact, these problems are in part responsible for the acceleration in development of nonviral strategies in the last years.

Most nonviral gene transfer technologies are employed for gene immune therapy or DNA vaccination studies. These studies are geared towards the introduction of DNA constructs through which proteins or peptides involved in cell-mediated immune responses or recombinant antibody production in the host are expressed. For intradermal or intramuscular applications, such as DNA vaccination approaches, the use of naked DNA has proven to provide efficient vaccines against different viral infections (such as hepatitis virus, influenza virus) or cancer vaccines in numerous animal models [11–15].

Over a decade ago, Wolff and coworkers demonstrated that the easy and simple needle and syringe injection of naked DNA is sufficient for *in vivo* gene transfer, resulting in the expression of the transgene [1]. However, despite the fact that this simple needle injection is sufficient to transduce naked DNA into muscle, this technique was largely inefficient for other tissue types, including tumors, and this is one important reason why numerous studies are dealing with the modification of this procedure for the improvement of transfer efficiencies [16–19]. These efforts have resulted in the development of, for example, the hydrodynamics-based procedure to deliver large volumes (more than 1 mL) of solutions containing naked DNA, which are either injected directly into the tissue or applied by intravenous injection over short times of only a few seconds [20, 21]. Although the efficiency of this procedure has been shown in several *in vivo* studies, at the current stage it seems rather restricted, to the perfusion of specific organs or particular portions of the desired organ as shown for the liver or kidney [21].

The gene gun, or particle bombardment technique, is based on the acceleration of DNA-coated gold or tungsten microparticles for gene transfer into different tissues. Because of its technical characteristics, however, this ballistic gene transfer of plasmid DNA achieves only limited penetration and so does not reach deeper areas of the targeted tissues. This is the reason why most studies using particle bombardment for nonviral gene transfer are aimed towards DNA vaccination or immunstimulatory approaches by targeting of antigen-presenting cells (APCs) in dermal and subdermal areas [22]. Currently many studies are favoring combinations of these technologies to improve *in vivo* gene transfer efficiencies significantly. Needle injection has been combined with *in vivo* electroporation or focused ultrasound in this context, for example [8, 23, 24].

Jet injection, initially reported as a novel method for injecting insulin in a needleless fashion [25], has developed into an applicable technology, allowing gene transfer into different tissue types with deeper penetration of the applied naked DNA. Thanks to technological improvements in this method it is now possible to achieve transfer efficiencies comparable to those of *in vivo* electroporation or particle bombardment [10, 26]. Jet injection technology is based on the use of high velocity fluid jets, possessing the required energy to penetrate skin and underlying tissues, resulting in the efficient transfection of the jet-injected tissue areas (Figure 7.1 C) [26]. The necessary acceleration of the fluid-jets is accomplished either through spring-forced systems or by application of pressurized air [27].

The low volume Swiss-Injector (EMS Medical, Nyon, Switzerland) utilizes compressed air to eject small volumes (3 to 10 μL) of solutions containing naked DNA into the target tissue at high speed (> 300 m · sec^{-1}). The energy of this accelerated liquid jet allows precise and effective penetration into tissue with a spread distribution of the liquid (Figure 7.1). The jet injection-mediated gene transfer covers broad areas associated with penetration of 5 to 10 mm within the jet-injected tissue (Figure 7.1 C). The design of the Swiss-Injector enables repeated jet injections at different pressures with one single filling of up to 200 μL. The volume of jet-injected fluids is positively correlated with the pressure used for jet injection, so higher pressure is used to apply larger volumes.

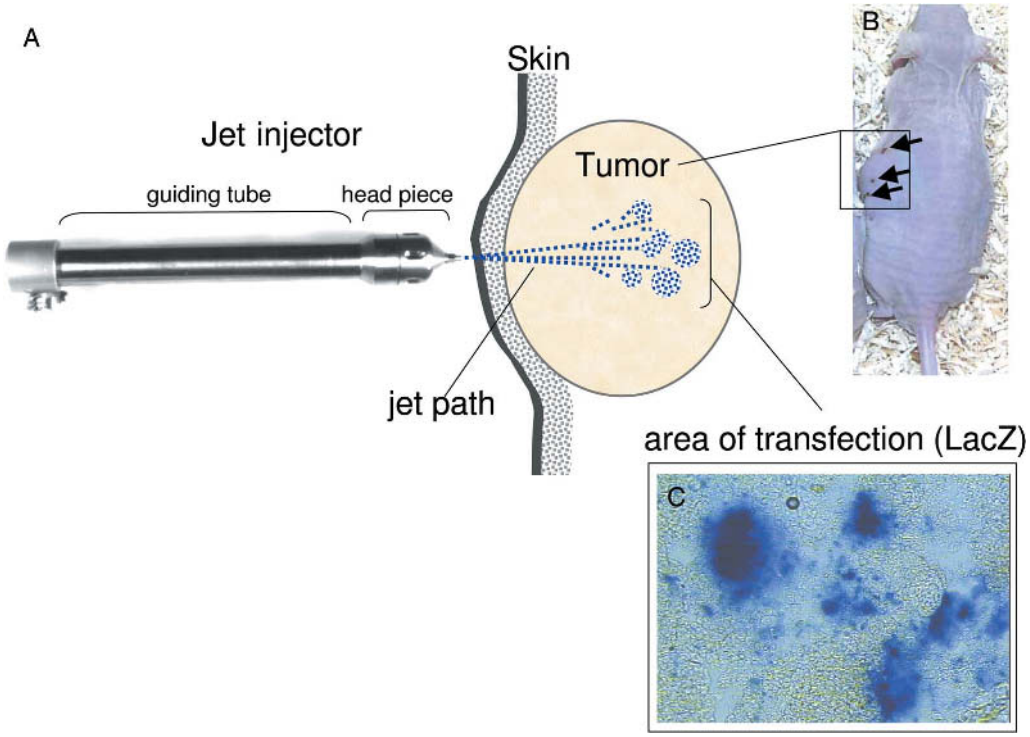

Figure 7.1 Schematic representation of the Swiss-Injector (A) and its use for intratumoral *in vivo* gene transfer. A projectile within the guiding tube of the jet-injector is accelerated by a pulse of compressed air hitting a piston, and this then transmits the impact to the liquid inside the compression chamber in the head piece. This results in ejection of small volumes (3 to 10 µL) through the nozzle, resulting in jet-penetration through the skin of the animal and into the tumor tissue for gene transfer (A, B). Jet injection of the LacZ-reporter gene expressing pCMVβ naked plasmid DNA gives rise to the scattered expression seen in the tumor tissue, which can be visualized as blue staining after the X-gal staining of cryosections of transduced tumors (C).

Although the naked DNA is exposed to strong physical forces during the jet injection, analyses of ejected DNA revealed only minimal alterations in DNA integrity, without significant shearing [28]. The low-volume jet injection system used for *in vivo* gene transfer combines efficiency in transfer of naked DNA with reduced effort in DNA formulation, so naked DNA can be jet-injected either as a simple solution in water or in appropriate buffers. In addition, the Swiss-Injector system provides the potential for simultaneous application of more than only one DNA construct into one tissue for the expression of two or more different gene products.

In vivo jet injection, and also *in vivo* electroporation and gene gun technology, have been successfully used in DNA vaccination studies [14, 29–31]. Muscle or

skin have been the target tissues for gene transfer in the majority of these *in vivo* studies, although only a small proportion of these studies were directed towards direct *in vivo* gene transfer into tumor tissue [32, 33]. Previous detailed studies have demonstrated the *in vivo* applicability of this jet injection device in different syngeneic mouse tumor models (B16 melanoma, Lewis lung carcinoma) and in xenotransplant models of human colon and mammary carcinomas [28, 34, 35].

This chapter summarizes data generated for the establishment and use of the hand-held low-volume Swiss-Injector prototype for efficient intratumoral *in vivo* jet injection gene transfer of naked DNA

7.2 *In vivo* Application of Jet Injection

7.2.1 Intratumoral Jet Injection of Naked Plasmid DNA

The Swiss-Injector prototype has been tested in several studies using syngeneic mouse (B16 malignant melanoma, Lewis lung carcinoma) and in xenotransplanted human tumor models (colon and mammary carcinoma) for the establishment of efficient gene transfer conditions. For evaluation of the feasibility of this technology, the studies in mouse and more importantly in xenotransplanted human tumor models are of particular interest if clinical use is anticipated.

To establish tumors, either 1×10^7 mouse tumor cells or xenotransplants of human colon carcinoma, derived from early passages of patient-derived tumors, were grown subcutaneously on mice to an approximate tumor size of 6×6 mm (Figure 7.1 B). Approximately 200 μL of a sterile DNA solution of the β-galactosidase (LacZ) expressing reporter plasmid pCMV-β or the GFP-expressing (GFP = green fluorescence protein) pEGFP-N1 vector were filled into the chamber of the jet injector head, and then jet-injected in small portions into the tumor. For the intratumoral gene transfer, four to five jet injections were applied for each tumor-bearing animal through the skin directly into the tumor tissue at a pressure of 3.0 bar, determined to be the most effective pressure for gene transfer (Figure 7.1 A, B). This particular application schedule supplies a total DNA dose of 40 to 50 μg DNA per animal, if a plasmid DNA concentration of 1 $\mu g \cdot \mu L^{-1}$ is used for gene transfer. The animals were anesthetized during the jet injection. Since the volumes of injected DNA solutions were relatively small, the injected fluid retained within the tumor tissue, and only minor bleeding occurred at the jet injection site.

In our jet injection studies, animals were kept for 24 to 120 hours after jet injection before sacrifice for tumor removal and further analyses of gene expression. Tumors were excised and shock-frozen in liquid nitrogen for subsequent preparation of cryosections for histochemical analysis, reporter gene assays of LacZ or GFP expression, or for the expression of other therapeutically relevant genes, such as human tumor necrosis factor alpha (TNF-α).

7.2.2
Analysis of Reporter Gene Expression in Jet-Injected Tumors

To localize LacZ expression in the jet-injected tumor tissues, direct staining of cryosections with X-gal staining was performed. To detect LacZ expression in the jet-injected tumors, tissues were cryosectioned and fixed in 2% formaldehyde. For the X-gal staining, slides were covered with X-gal solution and incubated at 37 °C for development of blue staining of the LacZ-transduced areas. The slides were covered with the Faramount aqueous mounting medium and evaluated under a light microscope (Figure 7.2 A, B).

For the detection of GFP expression in the pEGFP-N1 jet-injected tumors, the tissues were also cryosectioned and fixed in 2% formaldehyde, covered, and evaluated under a fluorescence microscope (Figure 7.2 C, D).

Figure 7.2 A and B show the staining for LacZ expression, which is scattered over a broad area of the jet-injected tissue of Lewis lung carcinoma. LacZ gene expression was already detectable in the jet-injected tumor tissue as early as 24 hours after jet injection, although strongest gene expression started 48 hours after jet injection. Similar expression kinetics have been detected in tumors jet-injected with the GFP-expressing pEGFP-N1 plasmid [34, 35]. The higher magnification provides a detailed view of the blue-spotted pattern of LacZ expression, with variations in intensities pointing to differences in the LacZ expression level. Our earlier quantitative analyses of LacZ expression by enzyme-linked immunosorbent assay (ELISA) have shown that these differences depend on the amount of naked DNA introduced in jet-injected tumor cells and also on the time of duration of reporter gene expression after gene transfer [34, 35]. Similar observations were made when the GFP-expressing pEGFP-N1 plasmid was jet-injected, resulting in bright fluorescence in the tumor 24 to 48 hours after jet injection (Figure 7.2 C, D).

Besides the use of reporter gene expressing plasmids in previous *in vivo* studies, the expression of jet-injected human TNF-α-expressing plasmid DNA was analyzed at different times after jet injection in xenotransplanted human colon carcinoma models, with high levels of the cytokine being detectable in these pCMV-hTNF jet-injected tumors 24 hours after gene transfer. After 48 hours the TNF-α expression had increased further, reaching a maximum 72 hours after jet injection. The level of cytokine expression remained at almost the same expression level during the observation time of 120 hours after jet injection. Comparable expression kinetics were observed in a Lewis lung carcinoma model after intratumoral jet injection of a TNF-expressing vector [35]. Jet injection gene transfer thus ensures efficient expression of the therapeutic cytokine gene for several days. This might represent a duration of transgene expression sufficient for effective therapeutic intervention.

Notably, we demonstrated in other *in vivo* studies that the simultaneous jet injection of the LacZ-expressing pCMVβ plasmid and the human TNF-α-expressing pCMV-hTNF plasmid results in the efficient expression of both gene products in the same tumor, underlining the effectiveness and versatility of this gene transfer technology [35].

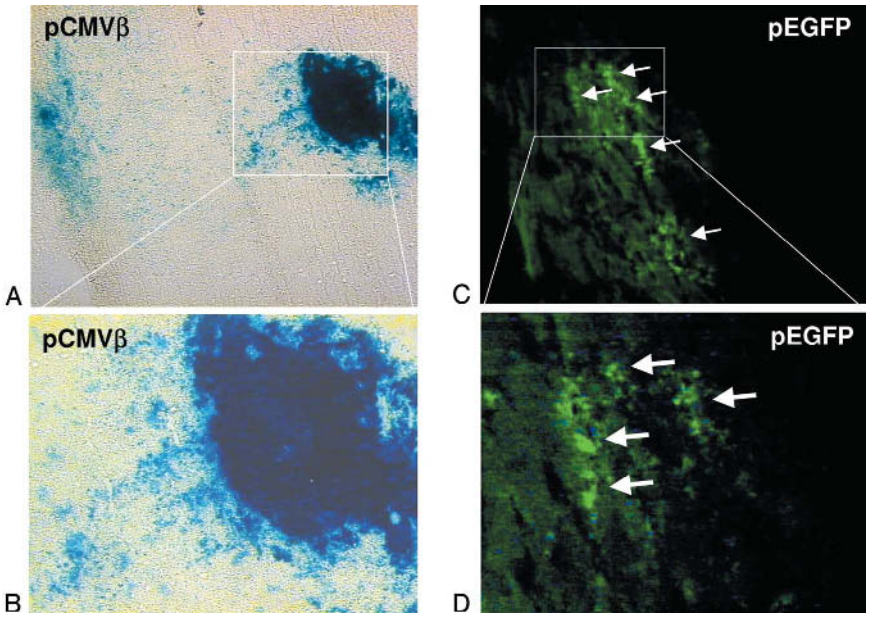

Figure 7.2 LacZ expression (A, B) and GFP expression (C, D) in cryosections of Lewis lung carcinomas detected 48 hours after jet injection of the naked plasmids pCMVβ or pEGFP-N1 at plasmid concentrations of 1 μg mL^{-1} (magnification 100× in A and C, 200× in B and D). Panels A and B show the scattered LacZ expression in the tumor tissue detected by the blue X-gal staining of cryosections. Panels C and D depict fluorescence microscopy in the GFP-expressing pEGFP-N1 in cryosections of jet-injected tumor tissue, indicated by the appearance of the bright green fluorescent areas within the tumor. The white arrows indicate sites of GFP expression.

7.2.3
Analysis of the Stability of Jet-Injected Naked DNA

DNA stability is decisive for efficient gene transfer and foreign gene expression. Several reports have shown that the degree of preservation of plasmid conformation, particularly of supercoiled plasmid DNA, has an impact on gene transfer efficiency. Jet injection technology is based on the use of high pressures to eject the DNA-containing solution through the nozzle of the jet injector, which has a narrow diameter of only 0.3 mm. These conditions might in fact create physical stress for the circular plasmid molecules, which could result in damage to the DNA, so we were interested to see if shearing of the jet-injected plasmid DNA might occur.

Figure 7.3 shows agarose gel electrophoresis of control and jet-injected DNA exposed to different ejection pressures and clearly demonstrates that alterations of the plasmid DNA are apparent. Increases in jet injection pressures result in increases in levels of damaged DNA, reflected in the appearance of degraded DNA in the respective lanes. However, the portion of such damaged plasmid DNA is comparatively low. Our earlier quantitative analyses of jet-injected DNA by capillary

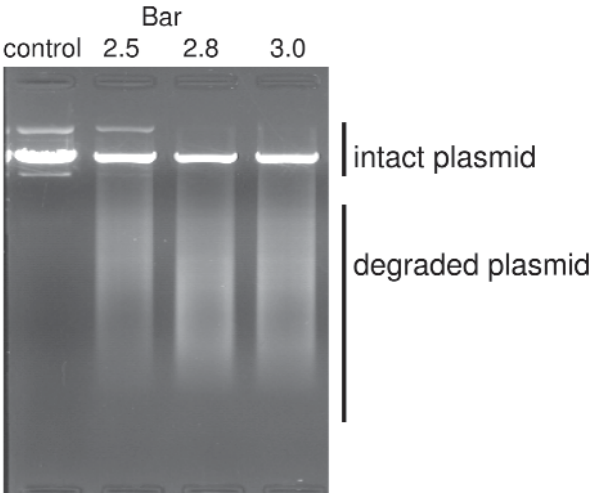

Figure 7.3 Evaluation of the impact on plasmid DNA of physical forces associated with jet injection, by agarose gel electrophoresis of the pCMVβ plasmid before and after jet injection. For agarose gel electrophoresis, the original DNA before filling (control) and samples from the ejected plasmid DNA were analyzed. The plasmid pCMVβ was ejected by the jet injector at pressures of 2.5 bar, 2.8 bar, and 3.0 bar. The intact plasmid DNA and the degraded portion of the plasmid are indicated.

gel electrophoresis (CGE) (see also Chapter 3) revealed that a maximum loss of 20% of the covalently closed circular (ccc) supercoiled form of the DNA occurred at the highest pressure setting of 3.0 bar [28, 35]. The CGE analysis further revealed that reduction in the jet injection pressure reduced the loss of the ccc form of plasmid DNA to less than 7%. However, studies in different tumor models have shown that higher jet injection pressures of 2.8 to 3.0 bar significantly improve the gene transfer efficiency, so conditions representing the optimal compromise between jet-pressure and preservation of intact DNA need to be defined for effective jet injection gene transfer.

7.3 Conclusions

This chapter describes the utilization of jet injection technology for gene transfer into tumors. Jet injection has been extensively tested for its feasibility for *in vivo* transfer of naked DNA and it has been demonstrated that it can be successfully employed for nonviral gene transfer [35–37].

Recent developments geared towards obtaining a suitable jet injection-based technology have resulted in the construction of the Swiss-Injector prototype, which requires only small amounts of naked plasmid DNA associated with a significant reduction in ejected volumes and improved accuracy and reproducibility of DNA

application. Serial measurements have revealed that the ejected volumes are constant, with minor variations of less than 10%.

The Swiss-Injector system used in the *in vivo* studies is capable of ejection of low volume jets for repeated naked DNA application into the targeted tissue, which is of advantage for *in vivo* applications to transduce larger tissue areas. With regard to the safety of the jet injection technology, we and others have observed no serious side effects in jet-injected animals [26, 27].

With respect to potential physical DNA damage by jet-associated shearing forces, our qualitative and quantitative analyses have revealed no significant loss of intact plasmid DNA. This finding is unquestionably of crucial importance for preservation of functional integrity for efficient foreign gene expression [28, 37].

In contrast to the majority of other studies, which employed jet injection technology for DNA vaccination and genetic immunization approaches, our experiments were aimed at direct intratumoral *in vivo* gene transfer. The efficient expression of the LacZ- and GFP-reporter genes and also of the therapeutic human TNF-α cytokine gene in the jet-injected tissue have been demonstrated. The pattern of transgene expression indicated that sufficient proportions of the tumor are affected, providing an expression level and a duration of expression sufficiently high to exert a therapeutic effect.

The data presented in this chapter and the results of our previous *in vivo* studies demonstrate that jet injection allows efficient gene expression through the application of small amounts of naked DNA in simple formulations. Previous findings that simultaneous jet injection of two different plasmids could result in the successful expression of both genes at the same jet injection site point to possible applications of combinations of different DNA constructs to achieve synergy of therapeutic genes transduced into the targeted tissue.

Overall, nonviral jet injection gene transfer of naked DNA has the potential for clinical application, particularly if local gene therapy approaches are anticipated in cancer treatment.

Acknowledgments

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References

1 Wolff, J. A., Malone, R. W., Williams, P., Chong, G., Acsadi, A., Jani, A., Felgner, P. L., Direct gene transfer into mouse muscle *in vivo*. *Science* **1990**, *247*, 1465–1458.

2 Herweijer, H., Wolff, J. A., Progress and prospects: naked DNA transfer and therapy. *Gene Ther.* **2003**, *10*, 453–458.

3 Li, S., Huang, L., Nonviral gene therapy: the promises and challenges. *Gene Ther.* **2000**, *7*, 31–34.

4 Nishikawa, M., Huang, L., Nonviral vectors in the new millenium: delivery barriers in gene transfer. *Hum. Gene Ther.* **2001**, *12*, 861–870.

5 Klinman, D. M., Conover, J., Leiden, J. M., Rosenberg, A. S., Sechler, J. M. G., Safe and effective regulation of hematocrit by gene gun administration of an erythropoietin-encoding DNA plasmid. *Hum. Gene Ther.* **1999**, *10*, 659–665.

6 Sikes, M. L., O'Malley, B. W., Finegold, M. J., Ledley, F. D., *In vivo* gene transfer into rabbit thyroid follicular cells by direct DNA injection. *Hum. Gene Ther.* **1994**, *6*, 837–844.

7 Yang, N.-S., Burkholder, J., Roberts, B., Martinell, B., McCabe, D., *In vivo* and *in vitro* gene transfer to mammalian cells by particle bombardment. *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 9568–9572.

8 Aihara, H., Miyazaki, J.-I., Gene transfer into muscle by electroporation *in vivo*. *Nat. Biotechnol.* **1998**, *16*, 867–870.

9 Somiari, S., Glasspool-Malone, J., Drabick, J. J., Gilbert, R. A., Heller, R., Jaroszeski, M., Malone, R. W., Theory and *in vivo* application of electroporative gene delivery. *Mol. Ther.* **2000**, *2*, 178–187.

10 Furth, P. A., Shamay, A., Wall, R. J., Hennighausen, L., Gene transfer into somatic tissue by jet injection. *Anal. Biochem.* **1992**, *205*, 365–368.

11 Vahlsing, H. L., Yankauckas, M., Sawdey, S. H., Gromkowski, M., Manthorpe, M., Immunization with plasmid DNA using a pneumatic gun. *J. Immunol. Methods* **1994**, *175*, 11–22.

12 Rakhmilevich, A. L., Turner, J., Ford, M. J., McCabe, D., Sun, W. H., Sondel, P. H., Grota, K., Yang, N.-S., Gene gun-mediated skin transfection with interleukin 12 gene results in regression of established primary and metastatic murine tumors. *Proc Natl. Acad. Sci. USA* **1996**, *93*, 6291–6296.

13 Liu, M. A., Ulmer, J. B., Gene based vaccines. *Mol. Ther.* **2000**, *1*, 497–500.

14 Macklin, M. D., McCabe, D., McGregor, M. W., Neumann, V., Meyer, T., Callan, R., Hinshaw, V. S., Swain, W. F., Immunization of pigs with a particle-mediated DNA vaccine to influenza A virus protects against challenge with homologous virus. *J. Virol.* **1998**, *72*, 1491–1496.

15 Turner, J. G., Tan, J., Crucian, B. E., Sullivan, D. M., Ballester, O. F., Dalton, W. S., Yang, N.-S., Burkholder, J. K., Yu, H., Broadened clinical utility of gene gun-mediated granulocyte-macrophage colony-stimulating factor cDNA-based tumor cell vaccines as demonstrated with a mouse myeloma model. *Hum. Gene Ther.* **1998**, *9*, 1121–1130.

16 Davis, H. L., Demeneix, B. A., Quantin, B., Coulombe, J., Whalen, R. G., Plasmid DNA is superior to viral vectors for direct gene transfer into adult mouse skeletal muscle. *Hum. Gene. Ther.* **1993**, *4*, 733–740.

17 Heinzerling, L., Feige, K., Rieder, S., Akens, M. K., Dummer, R., Stranzinger, G., Moelling, K., Tumor regression induced by intratumoral injection of DNA coding for human interleukin 12 into melanoma metastases in grey horses. *J. Mol. Med.* **2001**, *78*, 692–702.

18 Zhang, G., Vargo, D., Budker, V., Armstrong, N., Knechtle, S., Wolff, J. A., Expression of naked plasmid DNA injected into afferent and efferent vessels of rodent and dog livers. *Hum. Gene. Ther.* **1997**, *8*, 1763–1772.

19 Budker, V., Zhang, G., Danko, I., Williams, P., Wolff, J. A., The efficient expression of intravascularly delivered DNA in rat muscle. *Gene Ther.* **1997**, *5*, 272–276.

20 Liu, F., Song, Y. K., Liu, D., Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther.* **1999**, *6*, 1258–1266.

21 Zhang, G., Song, Y. K., Liu, D., Long-term expression of human alpha 1-antitrypsin gene in mouse liver achieved by intravenous administration of plasmid DNA using a hydrodynamics-based procedure. *Gene Ther.* **2000**, *7*, 1344–1349.

22 Pertmer, T. M., Eisenbraun, M. D., McCabe, D., Prayaga, S. K., Fuller, D. H., Haynes, J. R., Gene gun-based nucleic acid immunization: elicitation of humoral and cytotoxic T-lymphocyte responses following epidermal delivery of nanogram quantities of DNA. *Vaccine* **1995**, *13*, 1427–1430.

23 Wells, D. J., Gene Therapy Progress and Prospects: Electroporation and other physical methods. *Gene Ther.* **2004**, *11*, 1363–1369.

24 McCreery, T. P., Sweitzer, R. H., Unger, E. C., Sullivan, S., DNA delivery to cells *in vivo* by ultrasound. *Methods Mol. Biol.* **2004**, *245*, 293–298.

25 Weller, C., Linder, M., Jet injection of insulin vs the syringe-and-needle method. *JAMA* **1966**, *195*, 844–847.

26 Furth, P. A., Shamay, A., Hennighausen, L., Gene transfer into mammalian cells by jet injection. *Hybridoma* **1995**, *14*, 149–152.

27 Furth, P. A., Kerr, D., Wall, R., Gene transfer by jet injection into differentiated tissues of living animals and in organ culture. *Mol. Biotechnol.* **1995**, *4*, 121–127.

28 Walther, W., Stein, U., Fichtner, I., Voss, K., Schmidt, T., Schleef, M., Nellessen, T., Schlag, P. M., Intratumoral low volume jet injection for efficient nonviral gene transfer. *Mol. Biotechnol.* **2002**, *21*, 105–115.

29 Heansler, J., Verdelet, C., Sanchez, V., Girerd-Chambaz, Y., Bonnin, A., Trannoy, E., Krishnan, S., Meulien, P., Intradermal DNA immunization by using jet-injectors in mice and monkeys. *Vaccine* **1999**, *17*, 628–638.

30 Sawamura, D., Ina, S., Itai, K., Mex, X., Kon, A., Tamai, K., Hanada, K., Hashimoto, I., *In vivo* gene introduction into keratinocytes using jet injection. *Gene Ther.* **1999**, *6*, 1785–1787.

31 Seigne, J., Turner, J., Diaz, J., Hackney, J., Pow-Sang, J., Helal, M., Lockhart, J., Yu, H., Feasibility study of gene gun mediated immunotherapy for renal cell carcinoma. *J. Urol.* **1999**, *162*, 1259–1263.

32 Yamashita, Y., Shimada, M., Hasegawa, H., Minagawa, R., Rikimaru, T., Hamatsu, T., Tanaka, S., Shirabe, K., Miyazaki, J., Sugimachi, K., Electroporation-mediated interleukin-12 gene therapy for hepatocellular carcinoma in the mice model. *Cancer Res.* **2001**, *61*, 1005–1012.

33 Hui, K. M., Chia, T. F., Eradication of tumor growth via biolistic transformation with allogeneic MHC genes. *Gene Ther.* **1997**, *4*, 762–767.

34 Walther, W., Stein, U., Fichtner, I., Schlag, P. M., *In vivo* gene transfer of naked DNA into xenotransplanted colon carcinoma by jet injection. *Langenbeck's Archives Surg.* **2001**, *30*, 69–72.

35 Walther, W., Stein, U., Fichtner, I., Malcherek, L., Lemm, M., Schlag, P. M., Non-viral *in vivo* gene delivery into tumors using a novel low volume jet injection technology. *Gene Ther.* **2001**, *8*, 173–180.

36 Ren, S., Li, M., Smith, J. M., DeTolla, L. J., Furth, P. A., Low-volume jet injection for intradermal immunization in rabbits. *BMC Biotechnol.* **2002**, *23*, 10.

37 Cartier, R., Ren, S. V., Walther, W., Stein, U., Lewis, A., Schlag, P. M., Li, M., Furth, P. A., *In vivo* gene transfer by low-volume jet injection. *Anal. Biochem.* **2000**, *282*, 262–265.

8
Plasmid Inhalation: Delivery to the Airways

Lee A. Davies, Stephen C. Hyde, and Deborah R. Gill

8.1
Introduction

Gene transfer to the airways is being investigated as a possible therapy for a variety of acute and chronic lung diseases, such as cancer, cystic fibrosis, and emphysema. Lung gene transfer is also being developed for applications such as the prevention of lung transplant rejection and the treatment of lung damage after radiotherapy.

The lung is a complex organ containing multiple cell types. The tracheobronchial tree extends from the trachea down through numerous divisions of airways lined with epithelium. In the larger airways the epithelium is pseudostratified, consisting mainly of ciliated and non-ciliated columnar cells, goblet cells, and a layer of basal cells; the epithelium eventually transitions to a single layer of cells lining the respiratory bronchioles [1]. The parenchyma of the lung contains the gas-exchanging alveolar cells and is highly vascularized with capillary endothelial cells [2]. Therapeutic gene transfer will require gene expression in the appropriate cell types of the lung, which can be achieved by judicious selection of the gene transfer agent (GTA) (see also Chapter 5). Viral vectors transduce cells depending on receptor specificity and availability for uptake, although this may be modified by manipulation of the virus pseudotype. Several viral GTAs have given rise to debilitating immune and inflammatory responses after gene transfer to the lung, limiting the options for repeated administration of these vectors [3, 4]. Recently there has been increased interest in the use of non-viral, plasmid-based GTAs that can be manipulated to transfect a wide range of lung cell types [5]. In addition to the use of naked plasmid DNA (pDNA), plasmids may also be complexed with a variety of lipids, polymers, and polycations, and many of these have been evaluated after delivery to the lung [6].

8.2 Delivery Methods

Delivery to the lung is complex. The simplest method of delivering GTAs is by direct injection to local areas of the lung. Gene transfer of Vaccinia virus expressing IL-2, for example, was detected after injection into the chest walls of patients with malignant mesothelioma [7]. For more widespread gene transfer, the extensive vascularization of the lung suggests that systemic delivery of GTAs could be an option: systemic delivery of pDNA/liposome complexes to mice, for example, resulted in rejection of pulmonary metastases through non-specific increases in IL-12 expression [8]. Unmethylated CpG motifs present in bacterial DNA delivered in plasmids [9] are known to contribute to non-specific antitumor responses [10] and this approach may be further exploitable. However, systemic delivery in animal models mainly results in gene transfer to the pulmonary endothelium, or to localized areas of the lung parenchyma. To transfect epithelial cells, the GTA must escape from the capillaries and diffuse through layers of adjacent tissue, and reports of consistent gene transfer throughout the conducting airways by this method are rare. In one study, bronchial epithelial cells and submucosal glands were successfully transfected [11], but this was not the case in many studies with other GTAs. In addition, systemic delivery to the lung may be relatively inefficient, due to a high proportion of the administered non-viral GTA being delivered to other organs such as the liver during circulation.

8.2.1 Lung Delivery by Instillation

Consistent gene transfer of the airway epithelia appears to require topical delivery, which in many small animal models may be achieved by instillation of a bolus of fluid, resulting in transfection of the nasal and lung epithelia. The respiratory epithelia lining the nose and lung are similar and share many cell types in common, so the nasal epithelium has often been used as a surrogate tissue for the lungs. Controlled perfusion of the nasal epithelium may increase the contact of the GTA with the respiratory cells, maximizing the opportunity for gene transfer and avoiding some of the lung clearance mechanisms. The murine nasal epithelium has been used to test a variety of non-viral GTAs for evidence of functional gene transfer in transgenic cystic fibrosis (CF) mice [12, 13]. In the clinic, plasmid DNA complexed with the lipids DOTMA/DOPE has been used to express human α-1-antitrypsin (AAT) in the nasal epithelium of patients with AAT deficiency [14]. Perfusion of single and multiple doses of plasmid DNA complexed with the lipids DC-Chol/DOPE to the nasal epithelium have demonstrated functional gene transfer in CF patients and provided proof of concept for CF lung gene therapy [15, 16]. In one study, perfusion of naked plasmid DNA was at least as effective as plasmid DNA complexed with GL67 liposomes [17].

For delivery to the conducting airways of the lung, many preclinical studies with small animal models have used intranasal sniffing (insufflation) [18], or direct

intratracheal injection [19]. Any GTA that can be formulated as a liquid can be delivered directly by injection into the trachea. Both naked DNA and pDNA complexed with DOTMA/DOPE resulted in reporter gene expression in the mouse lung [20]. Insertion of a catheter via the trachea facilitated delivery to a single bronchus in rats [21]. Factors affecting the success or otherwise of non-viral GTAs by this delivery route also include the delivery vehicle, which impacts on transgene expression levels [21]. Tracheal delivery offers a relatively straightforward delivery route in small animals, requiring only a minor surgical procedure, while in larger animals a bronchoscope may be used for delivery to a defined area of the lung. Both naked DNA and pDNA complexed with GL67 liposomes have been successfully delivered to individual lobes of the sheep lung with detectable reporter gene expression [22]. Similarly, β-galactosidase reporter expression was detected in pigs after bronchoscopic delivery of plasmid DNA complexed with Lipofectin and an integrin-binding peptide [23]. Although the viscosity and volume of the final dose must be taken into consideration, these methods ensure that the majority of the GTA is delivered to the lung with little loss or release of material into the environment. Consequently, these approaches are suitable for initial gene transfer or toxicity studies where only small amounts of GTA material are available.

Apart from intranasal sniffing, which is relatively non-invasive, topical delivery procedures typically require anesthesia. The delivery of a large volume of liquid may have several drawbacks, including non-uniform distribution [24] and pooling of liquid in the lung parenchyma [22]. Bronchial instillation of pDNA complexed with the cationic polymer 22 kDa polyethylenimine (PEI) into rats resulted in severe inflammation and a reduction in lung function, which was significantly less marked for naked DNA, suggesting GTA-specific effects [25]. Moreover, the clearance of large volumes of liquid from the lungs may have unknown effects on gene transfer levels. Where limited material is available, a more uniform distribution can be achieved with coarse aerosols such as those generated with the Penn–Century MicroSprayer™ (Penn–Century Inc., Philadelphia, PA); by this approach adeno-associated virus was delivered to the lungs of Rhesus macaques by bronchoscope, resulting in 93% of the aerosol material being retained in the lung [26]. The large (15–30 μm) droplets generated by this method resulted in regional deposition, but avoided excessive parenchymal pooling.

8.2.2
Delivery by Aerosol

A far more appropriate technique for the topical delivery of GTAs to the respiratory tract is by aerosol. Inhalation therapy, in one form or another, has been practiced for centuries, and inhalation is now the primary route of pharmaceutical administration for respiratory diseases such as emphysema, asthma, and cystic fibrosis. Large volumes of fluid can be atomized quickly and inhaled by patients to provide direct access to the vast airway surface of the lung. Aerosol delivery thus maximizes the concentration of GTA in the lung, whilst reducing the risks associated with

systemic delivery to non-target organs and minimizing gene transfer to the germ line. The technique is non-invasive, does not require anesthetic, and is generally well tolerated by patients, such that repeated application for the treatment of chronic lung conditions is entirely feasible. Since many of the target cell types in the lung are terminally differentiated, the ability to aerosolize GTAs repeatedly is a key factor for successful gene therapy of chronic lung conditions.

8.2.3 Aerosol Deposition

The clinical benefits of any pharmaceutical agent delivered by aerosol will depend largely on the dose and distribution of the aerosol within the lung. Inappropriate targeting of drugs can result in reduced clinical efficacy [27] or in a number of unwanted side effects [28]. The exact site and quantity of aerosol deposition within the lung will be determined by a number of factors, including the nature of the aerosol itself and various respiratory parameters. The upper airways, particularly the nasal passages, work as an effective filtration system to remove unwanted airborne pathogens and contaminants, and in order to reach the lung, a therapeutic aerosol must overcome this filtration process. One way of increasing deposition is to bypass the nose completely by oral delivery of pharmaceutical agents. This has been shown to increase lung deposition of inhaled therapeutics significantly [29] and is the preferred route of delivery for inhaled drugs in humans. Aerosol deposition will also be affected by the anatomy of the respiratory tract; the size and branching of the conducting airways, as well as the depth and rate of breathing, will all affect where aerosols deposit [30]. Considerable increases in lung deposition can be achieved in humans by introducing a breath-hold maneuver at the end of an inhalation, as the aerosol then has more time in the lung to deposit by sedimentation [31]. The disease status of the patient will also have an effect on deposition, with constriction of airways and limited lung function both contributing to reduction or redistribution in lung deposition. One of the most important factors in determining lung deposition is the size of the aerosol particles, and studies in numerous species have demonstrated a correlation between the zone of deposition within the lung and particle size [32]. In humans, larger droplets (> 10 μm) are efficiently removed by inertial impaction in the mouth and fail to enter the lower airways. Smaller particles penetrate further into the lungs and can be deposited in the trachea and bronchi, but significant pulmonary deposition is only achieved with particles less than 5 μm in diameter [33].

8.2.4 Aerosolization Devices

Several technologies for the generation of pharmaceutical aerosols are currently in use. Aerosols produced by medical devices typically contain a heterogeneous population of particles with different physical diameters and not all particles will be small enough to penetrate into the lower airways.

8.2.4.1 Metered Dose Inhalers

The pressurized metered dose inhaler (MDI) (Figure 8.1) is currently the most popular form of respiratory drug delivery system. MDIs are small, inexpensive, and self-contained, making them ideal for delivery of a number of pharmaceutical agents. Inside the device, drug is suspended along with surfactants and preservatives in a volatile liquid propellant. Upon activation of the device a regulated dose of drug suspension is forced through a tiny spray orifice by vaporization of the propellant and a coarse aerosol of drug is generated.

Despite their popularity, the development of MDIs for the aerosol delivery of GTAs has been restricted by formulation requirements and by the very low delivery volumes associated with the devices. Agents to be aerosolized must be compatible with the high concentrations of propellant and surfactants within the spray formulation and as a result solvent-sensitive molecules such as some GTAs can be difficult to formulate for MDIs [34]. In addition, with typical aerosol doses of 25 μl to 100 μl per actuation it is most unlikely that MDIs could be used for the delivery of the large quantities of GTAs that may be required for a therapeutic effect [35] – a concern further exacerbated by the relative inefficiency of MDI aerosol delivery to the lung. High droplet velocities associated with the atomization process result in considerable drug impaction in the oropharynx, and only around 10% of aerosolized material actually reaches the lungs [36]. Although MDIs appear to have limited utility for gene therapy in humans, successful aerosolization of at least one GTA with an MDI has been demonstrated. Aerosolization of pDNA conjugated with the cationic lipid Lipofectamine (Life Technologies, Gaithersburg, MD) was shown to produce β-galactosidase reporter gene expression in the lungs of mice exposed to multiple actuations of a MDI device [37]. However, the technical limitations of

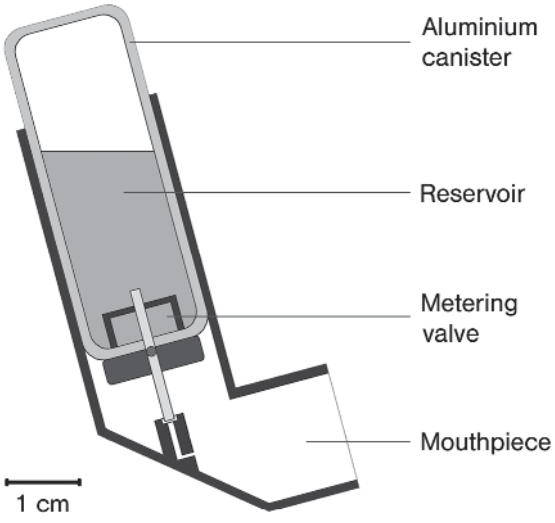

Figure 8.1 Schematic diagram of a pressurized metered dose inhaler.

MDI delivery for GTAs were illustrated by the fact that only 400 ng of DNA could be aerosolized with each activation of the device.

8.2.4.2 **Dry Powder Inhalers**

An alternative device for respiratory drug delivery is the dry powder inhaler (DPI). These share many of the practical advantages of the pressurized MDI, being quick to use, small, and portable, but utilize powdered drug instead of drug suspensions to create therapeutic aerosols (see also Chapter 10). Drug is loaded into the inhaler within a capsule or blister, and aerosol is generated by air turbulence as the patient inhales, drawing the powder through a plastic mesh or grid, thus breaking up larger particles and ensuring adequate dispersion of the aerosol. Whilst the DPI is a relatively simple device, the formulation and development of suitable drug powders for delivery is a lengthy and expensive process. Lung deposition requires drug particles to be from 1–5 μm in diameter [33], but at this small size, adhesive interparticle forces result in poor aerosol dispersion. Thus, to produce an aerosol of suitable quality for respiratory delivery, dry powder aerosols must be formulated with carrier molecules such as lactose to aid dispersion. Unfortunately, such carrier molecules may influence drug function, and optimization of dry powder formulations can be problematic. In theory, DPIs could be used to deliver high concentrations of GTAs rapidly to the lung, but for gene therapy applications their use has been hampered by inability to produce suitable GTA/carrier molecule dry powder formulations. Stable respirable aerosols of the cationic lipid GL67 have been reported [38] but more recent developments have seen progress through the use of lipid/polycation/DNA (LPD) complexes. Spray-drying of DOTAP, protamine sulfate, and pDNA formulations in the presence of lactose as a preservative produces stable dry powder LPD complexes that retain transfection efficiency even after storage for three months [39]. The generated LPD particles demonstrated appropriate characteristics for aerosol delivery, being spherical with a mean diameter of only 4 μm. When tested in a DPI, however, these formulations were poorly dispersed, with the majority of powder being retained within the device [40]. The dispersion of spray-dried LPD formulations was greatly improved by the addition of 0.3% leucine into the spray formulation but this reduced the overall transfection efficiency of the LPD complexes [40]. Whilst dry powder formulations have considerable potential for gene therapy in the lung, the issue of formulation remains a major obstacle to their practical use.

8.2.4.3 **Nebulizers**

Medical nebulizer devices are physically much larger than the MDI or DPI and are more commonly used in the hospital environment or for treatment in the home. Nebulizers generate aerosols from an internal reservoir, containing drug in the form of a fine suspension or solubilized in a liquid solvent such as water or saline. Many nebulizers are well suited to the delivery of large volumes of therapeutic agent that may be administered over an extended period of time. There are several types of medical nebulizer currently available, but the most common are the jet nebulizer and the ultrasonic nebulizer.

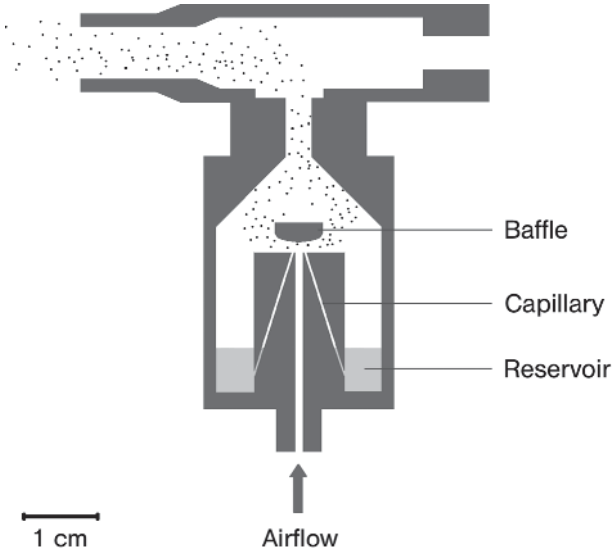

Figure 8.2 Schematic diagram of a jet nebulizer.

Within the jet or "pneumatic" nebulizer (Figure 8.2), compressed air is forced through a small orifice or venturi to create a high velocity jet of air. The rapidly expanding air stream creates an area of low pressure at the mouth of the venturi, which is utilized to draw fluid from the nebulizer reservoir through one or more capillary "feed" tubes. Liquid leaving the capillary is directed into the high velocity air stream, and shear forces generated at the air/liquid interface result in liquid fragmentation and the formation of aerosol droplets.

Many of the droplets produced by this aerosolization process are too large for efficient lung delivery, and are removed from the generated aerosol by a series of internal baffles positioned downstream. Because of their higher inertia, large droplets impact on the baffles and are returned to the reservoir for re-nebulization. Impaction and recirculation of larger droplets in this manner accounts for over 99% of all aerosolized material in jet nebulizers [41], but ensures that the nebulizer output contains a high proportion of respirable droplets. Ultrasonic nebulizers create aerosols by utilizing high frequency sound waves (usually over 1 MHz) to break up the free surface of a liquid reservoir. The required mechanical energy is typically provided by a quartz/zirconium piezoelectric transducer, which vibrates at high frequency under the control of an alternating electric field. The vibrations are transmitted via a coupling liquid and membrane to the liquid in the nebulizer reservoir, and at sufficiently high frequencies, the surface of the liquid is transformed into a fountain or geyser, which emits a "fog" of droplets that constitute the useful aerosol. As in the jet nebulizer, an arrangement of internal baffles prevents the release of larger droplets and the functional aerosol is evacuated by an applied airflow.

Nebulizers have several major advantages for delivery of gene therapy agents. Unlike the situation for an MDI or DPI, formulation requirements for nebulizers are minimal and pharmaceuticals can be aerosolized in solution or as suspensions. In addition, the large volumes utilized in nebulizer reservoirs (typically 3–10 ml) mean that relatively large doses of GTA may be delivered quickly. Consequently, nebulizers have been the most popular devices for aerosol delivery of gene therapy formulations and have been used in a number of preclinical and clinical studies.

8.2.5 Aerosolization of Plasmid DNA

Naked pDNA has many features to commend it as a gene therapy agent for a variety of disease applications. It is straightforward to manipulate and to manufacture (Chapter 3) in large quantities and can be stored in a stable fashion for extended periods (Chapter 3) [42]. However, the development of naked pDNA for lung gene therapy has been severely hampered by the loss of efficacy after nebulization [43]. Conventional jet and ultrasonic nebulizers generate considerable air/liquid shear forces during aerosol production. Naked DNA is extremely sensitive to applied shear forces [44] and is rapidly degraded when aerosolized with either jet [43, 45] or ultrasonic [46] nebulizers, resulting in subsequent loss of transfection efficiency. Plasmid degradation is further compounded in these devices by the continuous recycling of material through the nebulizer reservoir [41], resulting in repeated exposure of DNA to shear damage and progressive degradation (Figure 8.3). Consequently, naked DNA is not currently a viable gene transfer agent for aerosol delivery. However, pDNA can be successfully aerosolized if protected from degradation by complexation with cationic lipids or cationic polymers (see also Chapter 5).

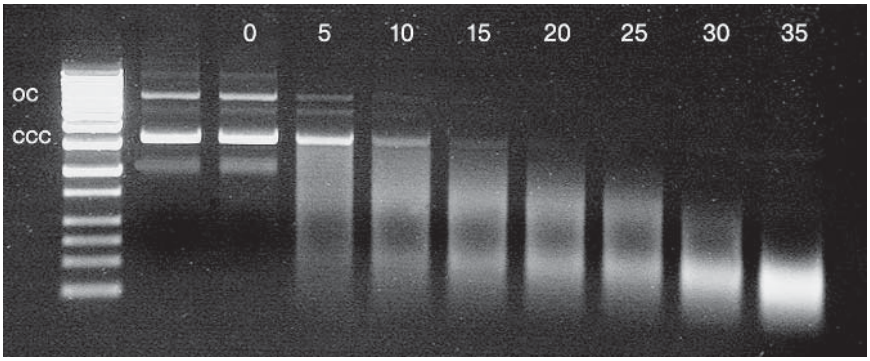

Figure 8.3 Degradation of naked plasmid DNA during jet nebulization. Plasmid DNA (5.6 kb) was aerosolized at 40 psi with an Aerotech II (CIS-US, Bedford, MA) jet nebulizer, with samples removed from the nebulizer reservoir at five-minute intervals for conformational analysis by gel electrophoresis. Aerosolization resulted in progressive degradation of plasmid DNA with loss of both covalently closed circular (ccc) and open circular (oc) plasmid forms. Lane 1 – Plasmid size markers, lane 2 – Reference plasmid (not aerosolized), lanes 3–10, plasmid DNA samples after 0–35 minutes of aerosolization.

8.2.6 Plasmid DNA/Lipid Complexes

Whilst nebulization of naked pDNA is associated with a dramatic loss in transfection efficiency, numerous studies have demonstrated gene transfer after aerosolization of pDNA complexed to cationic lipids. In one of the first studies to demonstrate gene expression *in vivo* after aerosol delivery of pDNA/lipids, 12 mg of plasmid DNA expressing the chloramphenicol acetyl transferase (CAT) reporter gene complexed with DOTMA/DOPE was aerosolized to the lungs of mice by jet nebulizer [47]. Significant levels of reporter gene activity were detected in the lungs of treated animals, and immunohistochemical analysis revealed widespread CAT expression in airway epithelial cells and alveolar lining cells. Since these groundbreaking studies, a number of groups have reported successful gene transfer into the lungs of mice [48, 49], rabbits [50], and Rhesus macaques [51] with a range of available cationic lipids. Although the vast majority of studies have utilized jet nebulizers for the aerosol delivery of DNA/lipid vectors, encouraging results have also been achieved with ultrasonic nebulizers. No loss of transfection efficiency was observed in rats instilled with aerosolized material after ultrasonic nebulization of pDNA complexed to DOTMA/Chol [46], and ultrasonic nebulization of two novel lipids – GLB73/DOPE and NL177/DOPE – was more recently shown to result in significant reporter gene expression in the lungs of mice [52]. Whilst considerable success has been achieved with aerosol delivery of pDNA/lipid formulations, the viability of aerosolized material has been shown to be highly dependent upon the specific cationic lipid used in the study. Aerosolization of pDNA complexed to the widely used lipids DCChol/DOPE and DMRIE/DOPE resulted in almost complete loss of transfection ability when aerosolized samples were used to transfect cells *in vitro,* but pDNA complexed to the lipid BGTC/DOPE retained over 80% of initial transfection efficiency under identical conditions [53]. It appears that the lipid formulation is important in determining the degree of plasmid protection during aerosolization, but the precise mechanism remains to be determined. In addition to the lipid formulation, the choice and operating characteristics of the nebulizer also have a significant impact on the transfection efficiency of aerosolized pDNA/lipid complexes [54]. This effect could be due to the variations in shear force and recycling time that occur within the reservoirs of different nebulizers. Consequently, aerosol delivery studies can be optimized by careful selection of both lipid and nebulizer.

8.2.6.1 Optimization of Aerosol Formulation

Although aerosol delivery has been investigated in large animal models, the majority of *in vivo* studies with pDNA/lipid complexes have used mice, largely for convenience and the availability of good disease models. However, it is difficult to deliver large amounts of material to the mouse lung by aerosol because very small aerosol droplets (< 1 μm in diameter) are required for significant lung deposition [55] and most commercial nebulizers generate droplets of 2–5 μm [56]. As a result, aerosol delivery of pDNA/lipid complexes to mice is very inefficient; studies with fluorescently

labeled lipid have shown that only 0.06% of material in the nebulizer reservoir was actually deposited in the lungs of exposed mice [47]. However, few studies have attempted to optimize formulations for aerosol delivery due to the large quantity of reagent required and the inherent cost associated with the aerosolization of DNA/lipid complexes.

In general, aerosol formulations have been determined on the basis of optimal results obtained *in vitro* or after instillation *in vivo*. However, studies with two cationic lipids – GL53 and GL67 (Genzyme Corp, Cambridge, MA) – revealed that optimal formulations for aerosol delivery were very different to those predicted by these methods [45]. Analysis of pDNA integrity after aerosolization with a Puritan Bennett Raindrop nebulizer (Puritan Bennett, Lenexa, KA) demonstrated that the degree of plasmid degradation during aerosolization of pDNA/lipid complexes correlated strongly with the extent to which the DNA was complexed with lipid. When pDNA/GL53/DOPE was aerosolized at high pDNA/lipid ratios, uncomplexed pDNA was quickly degraded, but when more lipid was added to the formulation the majority of pDNA remained intact after aerosolization [45]. When pDNA/GL67/DOPE was examined, an optimal pDNA/lipid ratio of 1 : 0.75 was found for aerosol delivery, compared to 1 : 0.25 used both *in vitro* and for instillation studies [18]. Modification of the instillation formulation was necessitated by the need for efficient pDNA transfer and protection from degradation during nebulization. In order to increase the potential rate of pDNA/lipid aerosol delivery, further modifications of the GL67 cationic lipid formulation were subsequently made to increase the concentration of pDNA in the aerosol formulation [49]. Formulations of pDNA with cationic lipid are colloidal in nature and are prone to aggregation and precipitation at higher concentrations [45]; a problem exacerbated in aerosol delivery by the propensity of jet nebulizers to aerosolize solvent preferentially [57], resulting in a concentration of pDNA/lipid vectors in the nebulizer reservoir. However, incorporation of the bilayer-stabilizing lipid DMPE-PEG_{5000} into GL67/DOPE formulations allowed production of stable pDNA/lipid complexes containing up to 6 mg/ml pDNA, ten times higher than had been previously reported [49]. This significant increase in concentration made delivery of large doses of lipid/pDNA to the human lung practical and resulted in the use of this formulation in the first aerosol study to deliver non-viral GTAs to the lungs of patients [35].

8.2.6.2 **Aerosol Delivery of Lipid/pDNA to Human Lung**

In order for clinical trials for lung gene therapy to begin, a safe, clinically feasible delivery system was required. Aerosol delivery to the lungs is minimally invasive, generally well tolerated, and may mitigate inflammatory side effects. In animal studies, instillation of pDNA/GL67 into mouse nose resulted in dose-dependent pulmonary inflammation with neutrophil and macrophage infiltration and elevation of interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) [58]. However, no corresponding histological toxicity was observed after aerosol delivery of pDNA/GL67 complexes to the lungs of mice [49] or Rhesus macaques [59]. The safety profile of aerosol delivery for lung gene transfer provided support for human lung trials, and in 1999, approximately 40 mg pDNA expressing the

human CFTR cDNA, complexed with GL67/DOPE/DMPE-PEG_{5000}, was aerosolized to each of eight cystic fibrosis patients using a PARI LC plus nebulizer (PARI Respiratory Equipment Inc, Richmond, VA); a further eight patients received the lipid alone [35]. The study was encouraging in terms of efficacy but also revealed some unexpected safety issues. Influenza-like symptoms were reported in seven out of eight patients in the active group, beginning 6 hrs after dosing and subsiding by 30 hrs, with slightly milder symptoms (increased cough and sputum) reported in three out of eight patients in the placebo group. These effects were replicated in a second study [60], but were not observed in an earlier safety study in normal volunteers [61]. Although not proven, it is thought that the pro-inflammatory effect of the pDNA/lipid complexes may be due to unmethylated CpG dinucleotide motifs present in the pDNA, and moves to generate clinical pDNA vectors with reduced numbers of these motifs are under way [62, 63].

8.2.7
Plasmid Delivery with Cationic Polymers

Most research into the aerosolization of non-viral gene transfer agents has focused on the use of cationic lipids to protect DNA during aerosolization, but recent studies have demonstrated the potential of the cationic polymer polyethylenimine (PEI) as a viable alternative. PEI exhibits a high cationic charge potential with considerable buffering capacity, and effectively complexes and compacts pDNA, providing high transfection efficiency both *in vitro* and *in vivo* [64]. Several forms of PEI are commercially available; both 22 kDa [65] and 25 kDa forms [66] have demonstrated significant levels of gene expression in the lungs of mice after instillation, but successful aerosolization studies have so far only been reported with the 25 kDa branched polymer. Jet nebulization of pDNA/PEI complexes resulted in only minimal loss of transfection efficiency [67]. In mice, aerosolized pDNA/PEI complexes produced high levels of expression in lung samples, despite the fact that only a relatively small dose of 1 mg of DNA was nebulized [67]. Indeed the measured levels of reporter gene expression were far higher than those observed when pDNA/lipid formulations were aerosolized to mice under the same conditions (Figure 8.4). Optimization of the aerosol delivery of pDNA/PEI to the mouse lung model demonstrated that a three-fold improvement in lung expression could be achieved when the compressed air used to generate aerosol included 5% carbon dioxide [68]. The elevated carbon dioxide levels probably caused the animals to increase their frequency of breathing and tidal volume, resulting in increased complex deposition in the lung. As with cationic lipids, the transfection efficiency of aerosolized pDNA/PEI reagents is dependent upon the ratio of gene transfer agent to pDNA in the aerosolized complex. In pDNA/PEI complexes the N/P ratio (where N represents positively charged nitrogen atoms and P represents negatively charged phosphates in the DNA backbone) is important in determining transfection efficiency both *in vitro* [67] and *in vivo* after instillation of pDNA/PEI complexes to the mouse lung [66]. Transfection efficiency after aerosolization of pDNA/PEI complexes has been shown to be optimal when the N/P ratio of aerosolized

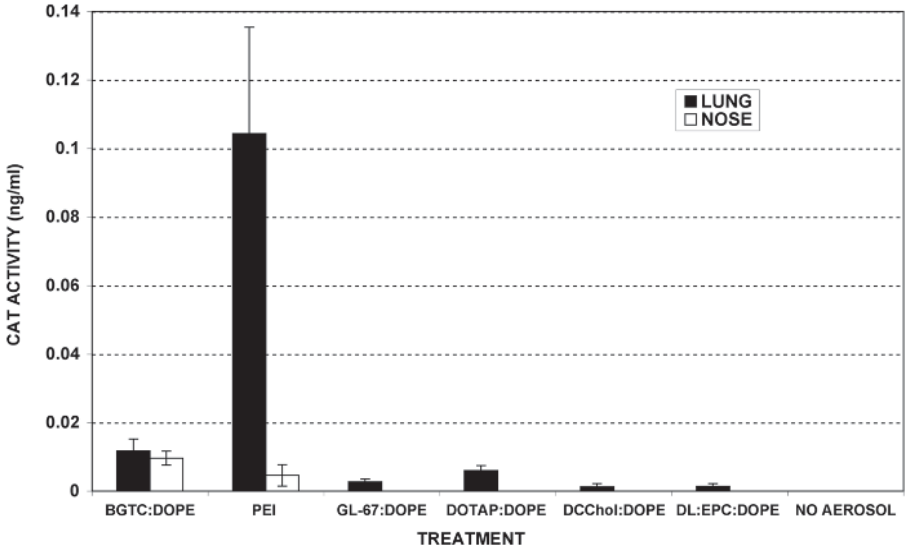

Figure 8.4 Relative *in vivo* efficacy of non-viral gene transfer agents delivered by aerosol. BALB/c mice were exposed to aerosols containing 2 mg of plasmid expressing the bacterial chloramphenicol acetyl transferase (CAT) reporter gene, conjugated to a range of non-viral GTAs. Lung and nasal tissue was harvested 48 hours after exposure and assayed for reporter gene expression. Aerosolization of plasmid DNA conjugated to the cationic polymer polyethylenimine (PEI) was shown to exhibit higher transfection levels than the cationic lipids tested. (Reproduced from [67], with permission).

complexes is between 10 : 1 and 20 : 1 [68]. Studies utilizing fluorescently labeled PEI in conjunction with immunohistochemical detection of reporter gene expression have demonstrated that pDNA/PEI complexes deposit upon and subsequently transfect the majority of epithelial cells in the conducting airway [69]. As a result, PEI aerosols have potential for use in the treatment of a variety of lung diseases, including cystic fibrosis and α-1-antitrypsin deficiency. However, most studies have focused on applications of PEI aerosols for the treatment of lung cancer.

Aerosol delivery of PEI complexed to pDNA expressing the p53 tumor suppressor gene showed significant reduction in tumor development in mouse lung cancer models after twice weekly aerosol exposure; treated animals also showed increased mean survival relative to control animals [70, 71]. Anti-tumor effects have also been reported after 6 weeks of twice weekly aerosol exposure of PEI complexed to pDNA expressing the murine IL-12 gene [72]. Whilst high levels of IL-12 expression could be detected in the lungs of treated mice, no IL-12 could be detected in plasma samples even after 6 weeks of treatment. Systemic delivery of IL-12 has been associated with severe toxic side effects in patients and consequently the highly selective expression of IL-12 in the lung after PEI aerosol delivery may provide a therapeutically beneficial option.

As with aerosol delivery of cationic lipids, aerosol delivery of pDNA/PEI complexes results in minimal toxicity in the mouse lung. Whereas instillation can result in

severe immune cell infiltration [66], aerosol administration of pDNA/PEI did not produce any histological changes in the lungs of treated mice [68]. Investigations into inflammatory cytokine responses have shown that both TNF-α and IL-1β were slightly elevated in the lungs and bronchoalveolar lavage fluid of mice exposed to PEI aerosols [73]. However, there was no increase in serum levels of these cytokines and, even in the lung, cytokine levels were much lower than when pDNA/lipid was aerosolized or when pDNA/PEI was administered by intravenous injection. These data demonstrate the relative safety of aerosol administration of pDNA/PEI vectors.

The encouraging results obtained in rodents have not so far been translated into clinical studies, although several groups are examining PEI aerosol delivery in large animal models. Preliminary deposition studies using radiolabeled technecium bound to pDNA/PEI complexes have demonstrated excellent bilateral distribution of complexes throughout the lungs of dogs, with around 10–20% of the aerosolized dose depositing within the lungs [74]. Plasmid DNA/PEI aerosols have also been successfully delivered to the lungs of anesthetized sheep by use of a PARI LC plus jet nebulizer in conjunction with a negative pressure ventilation system. In these studies, quantitative TaqMan PCR revealed consistent DNA deposition throughout the lungs, as well as detectable levels of reporter gene expression [75]. In addition, unlike cationic lipids, PEI-mediated gene transfer is not inhibited by the presence of pulmonary surfactant [76]. Together these results suggest that PEI aerosols have great potential for the administration of gene therapy vectors to the lung.

8.3 Future Directions

The delivery of gene therapy agents to the lung by aerosol is a relatively new field, yet considerable advances have been made both in vector design and in delivery techniques. Further progress will be required, however, before aerosol gene therapy can become part of a standard therapeutic regime. Current non-viral gene transfer agents are relatively inefficient for gene transfer and clinical gene therapy for the lung will require delivery of large volumes of vector. Not only is this material extremely expensive to produce, but delivery of such large volumes is also likely to require a considerable time with the associated inconvenience to the patient. The inefficiency of non-viral GTAs is further compounded by the general inefficiency of current aerosol delivery devices, which often deliver much less than 20% of the starting material to the lung [77].

A simple way to improve the performance of aerosolized GTAs would therefore be to increase the percentage of aerosolized material that deposits in the lung. The current inefficiency of aerosol delivery devices has been tolerated because available drugs have been inexpensive, but the ban on chlorofluorocarbon propellants in MDIs, along with the development of expensive inhalable therapies for topical and systemic lung delivery, have resulted in the development of a new generation of efficient aerosol devices. Electronic nebulizer devices that generate aerosols by means of a vibrating mesh will shortly become available for clinical use. Devices

such as the Aerodose inhaler (Aerogen, Mountain View, CA) or the I-Neb inhaler (Profile Therapeutics, Bognor Regis, UK) represent a considerable step forward in the efficient delivery of pharmaceutical agents to the lung. These devices generate aerosols with a high fine-particle fraction and have a significantly higher efficiency of delivering drug to the respiratory tract than conventional nebulizers [78]. Deposition studies using the Aerodose inhaler have shown that up to 85% of aerosolized material actually deposits in the lungs of patients, in comparison with 21% for patients treated with an MDI [79]. In addition, vibrating mesh nebulizers also have high aerosol output rates of 0.3–0.6 ml/min, minimizing the time required for aerosol delivery. These nebulizers now need to be tested with gene therapy agents.

One novel device that has been tested is the 'single pass' AERx® delivery system (Aradigm Corporation, Hayward, CA) that generates aerosol by extrusion of liquid under pressure through a nozzle array and can successfully aerosolize pDNA/lipid with no loss of transfection efficiency [80]. In the same study, aerosolization of naked DNA resulted in only minimal pDNA degradation relative to jet nebulization [43]. Another "single pass" aerosolization technique that may be suitable for the aerosolization of delicate GTAs such as naked DNA is electrohydrodynamic (EHD) comminution, which utilizes strong electric fields instead of air/liquid shear forces to break up bulk liquids into aerosols of fine droplets. The shear forces involved in EHD aerosol production are extremely small [81] and consequently even relatively large naked pDNA molecules (up to 15 kb) can be aerosolized with no visible loss of plasmid integrity (L. Davies, unpublished data). The Mystic™ drug delivery device (Battelle Pharma Inc, Columbus, OH) will be the first commercially available inhaler based on EHD technology and preliminary studies have demonstrated promising deposition levels of around 78% of aerosolized material in the lungs of volunteers [82].

8.4 Conclusions

Whilst considerable progress has been made in the field of aerosol delivery of genes to the lung over the last decade, there is clearly much work still to be done. Improvements in both vector design and aerosol administration will be required to deliver the true benefits of targeted gene expression in the lung. The new generation of nebulizer devices offers the possibility of delivering current GTAs with much greater efficiency than has been possible previously. In addition, the development of "single pass" and low shear nebulizers should allow studies into a whole range of new GTAs that were previously too fragile to deliver by aerosol. The potential benefits for lung gene therapy are enormous and if research continues at the current rate it is only a matter of time before aerosol gene therapy becomes an important aspect of medical intervention in respiratory disease.

References

1 Breeze, R. G., Wheeldon, E. B., The cells of the pulmonary airways. *Am. Rev. Respir. Dis.* **1977**, *116*, 705–777.

2 Crapo, J. D., Barry, B. E., Gehr, P., Bachofen, M., Weibel, E. R., Cell number and cell characteristics of the normal human lung. *Am. Rev. Respir. Dis.* **1982**, *126*, 332–337.

3 Yang, Y., Li, Q., Ertl, H. C. and Wilson, J. M., Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. *J. Virol.* **1995**, *69*, 2004–2015.

4 Halbert, C. L., Standaert, T. A., Aitken, M. L., Alexander, I. E., Russell, D. W., et al., Transduction by adeno-associated virus vectors in the rabbit airway: efficiency, persistence, and readministration. *J. Virol.* **1997**, *71*, 5932–5941.

5 Gill, D. R., Davies, L. A., Pringle, I. A., Hyde, S. C., The development of gene therapy for diseases of the lung. *Cellular and Molecular Life Sciences* **2004**, *61*, 355–368.

6 Ziady, A. G., Davis, P. B., Konstan, M. W., Nonviral gene transfer therapy for cystic fibrosis. *Expert Opin. Biol. Ther.* **2003**, *3*, 449–458.

7 Mukherjee, S., Haenel, T., Himbeck, R., Scott, B., Ramshaw, I., et al., Replication-restricted vaccinia as a cytokine gene therapy vector in cancer: persistent transgene expression despite antibody generation. *Cancer Gene Ther.* **2000**, *7*, 663–670.

8 Dow, S. W., Fradkin, L. G., Liggitt, D. H., Willson, A. P., Heath, T. D., et al., Lipid–DNA complexes induce potent activation of innate immune responses and antitumor activity when administered intravenously. *J. Immunol.* **1999**, *163*, 1552–1561.

9 Scheule, R. K., The role of CpG motifs in immunostimulation and gene therapy. *Adv. Drug Deliv. Rev.* **2000**, *44*, 119–134.

10 Lanuti, M., Rudginsky, S., Force, S. D., Lambright, E. S., Siders, W. M., et al., Cationic lipid/bacterial DNA complexes elicit adaptive cellular immunity in murine intraperitoneal tumor models. *Cancer Res.* **2000**, *60*, 2955–2963.

11 Koehler, D. R., Hannam, V., Belcastro, R., Steer, B., Wen, Y., et al., Targeting transgene expression for cystic fibrosis gene therapy. *Mol. Ther.* **2001**, *4*, 58–65.

12 Ziady, A. G., Gideon, C., Payne, J. M., Muhammad, O., Oette, S., et al., Stabilized PLASmin® complexes deliver exogenous genes to the nasal epithelia of cystic fibrosis (CF) knockout mice and confer partial physiological correction of CFTR-mediated chloride transport. *Mol. Ther.* **2002**, *5*, S435.

13 Ziady, A. G., Kelley, T. J., Milliken, E., Ferkol, T., Davis, P. B., Functional evidence of CFTR gene transfer in nasal epithelium of cystic fibrosis mice *in vivo* following luminal application of DNA complexes targeted to the serpin-enzyme complex receptor. *Mol. Ther.* **2002**, *5*, 413–419.

14 Brigham, K. L., Lane, K. B., Meyrick, B., Stecenko, A. A., Strack, S., et al., Transfection of nasal mucosa with a normal alpha1-antitrypsin gene in alpha1-antitrypsin-deficient subjects: comparison with protein therapy. *Hum. Gene Ther.* **2000**, *11*, 1023–1032.

15 Gill, D. R., Southern, K. W., Mofford, K. A., Seddon, T., Huang, L., et al., A placebo-controlled study of liposome-mediated gene transfer to the nasal epithelium of patients with cystic fibrosis. *Gene Ther.* **1997**, *4*, 199–209.

16 Hyde, S. C., Southern, K. W., Gileadi, U., Fitzjohn, E. M., Mofford, K. A., et al., Repeat administration of DNA/liposomes to the nasal epithelium of patients with cystic fibrosis. *Gene Ther.* **2000**, *7*, 1156–1165.

17 Zabner, J., Cheng, S. H., Meeker, D., Launspach, J., Balfour, R., et al., Comparison of DNA/lipid complexes and DNA alone for gene transfer to cystic fibrosis airway epithelia *in vivo*. *J. Clin. Invest.* **1997**, *100*, 1529–1537.

18 Lee, E. R., Marshall, J., Siegel, C. S., Jiang, C., Yew, N. S., et al., Detailed analysis of structures and formulations of cationic lipids for efficient gene transfer to the lung. *Hum. Gene Ther.* **1996**, *7*, 1701–1717.

19 Griesenbach, U., Chonn, A., Cassady, R., Hannam, V., Ackerley, C., et al., Comparison between intratracheal and intravenous administration of liposome/DNA complexes for cystic fibrosis lung gene therapy. *Gene Ther.* **1998**, *5*, 181–188.

20 Meyer, K. B., Thompson, M. M., Levy, M. Y., Barron, L. G., Szoka, F. J., Intratracheal gene delivery to the mouse airway: characterization of plasmid DNA expression and pharmacokinetics. *Gene Ther.* **1995**, *2*, 450–460.

21 Sawa, T., Miyazaki, H., Pittet, J. F., Widdicombe, J. H., Gropper, M. A., et al., Intraluminal water increases expression of plasmid DNA in rat lung. *Hum. Gene Ther.* **1996**, *7*, 933–941.

22 Emerson, M., Renwick, L., Tate, S., Rhind, S., Milne, E., et al., Transfection efficiency and toxicity following delivery of naked plasmid DNA and cationic lipid/DNA complexes to ovine lung segments. *Mol. Ther.* **2003**, *8*, 646–653.

23 Cunningham, S., Meng, Q. H., Klein, N., McAnulty, R. J., Hart, S. L., Evaluation of a porcine model for pulmonary gene transfer using a novel synthetic vector. *J. Gene Med.* **2002**, *4*, 438–446.

24 Brain, J. D., Knudson, D. E., Sorokin, S. P., Davis, M. A., Pulmonary distribution of particles given by intratracheal instillation or by aerosol inhalation. *Environ Res.* **1976**, *11*, 13–33.

25 Uduehi, A. N., Stammberger, U., Kubisa, B., Gugger, M., Buehler, T. A., et al., Effects of linear polyethylenimine and polyethylenimine/DNA on lung function after airway instillation to rat lungs. *Mol. Ther.* **2001**, *4*, 52–57.

26 Beck, S. E., Laube, B. L., Barberena, C. I., Fischer, A. C., Adams, R. J., et al., Deposition and expression of aerosolized rAAV vectors in the lungs of Rhesus macaques. *Mol. Ther.* **2002**, *6*, 546–554.

27 Ruffin, R. E., Montgomery, J. M. and Newhouse, M. T., Site of beta-adrenergic receptors in the respiratory tract: use of fenoterol administered bytwo methods. *Chest* **1978**, *74*, 256–260.

28 Davies, D. S., Pharmacokinetic Studies with Inhaled Drugs. *Eur. J. Respir. Dis.* **1982**, *63*, 67–72.

29 Chua, H. L., Collis, G. G., Newbury, A. M., Chan, K., Bower, G. D., et al., The influence of age on aerosol deposition in children with cystic fibrosis. *Eur. Respir. J.* **1994**, *7*, 2185–2191.

30 Pavia, D., Thomson, M. L., Clarke, S. W. and Shannon, H. S., Effect of lung function and mode of inhalation on penetration of aerosol into the human lung. *Thorax 3* **1977**, *2*, 194–197.

31 Dolovich, M., Ruffin, R. E., Roberts, R., Newhouse, M. T., Optimal delivery of aerosols from metered dose inhalers. *Chest* **1981**, *80*, 911–915.

32 Schlesinger, R. B., Comparative deposition of inhaled aerosols in experimental animals and humans: a review. *J. Toxicol. Environ. Health* **1985**, *15*, 197–214.

33 Clark, A. R., Egan, M., Modelling the Deposition of Inhaled Powdered Drug Aerosols. *Journal of Aerosol Science* **1994**, *25*, 175–186.

34 Vervaet, C., Byron, P. R., Drug/surfactant/propellant interactions in HFA-formulations. *Int. J. Pharm.* **1999**, *186*, 13–30.

35 Alton, E. W., Stern, M., Farley, R., Jaffe, A., Chadwick, S. L., et al., Cationic lipid-mediated CFTR gene transfer to the lungs and nose of patients with cystic fibrosis: a double-blind placebo-controlled trial. *Lancet* **1999**, *353*, 947–954.

36 Dolovich, M., Ruffin, R., Corr, D., Newhouse, M. T., Clinical evaluation of a simple demand inhalation MDI aerosol delivery device. *Chest* **1983**, *84*, 36–41.

37 Brown, A. R. and Chowdhury, S. I., Propellant-Driven Aerosols of DNA Plasmids for Gene Expression in the Respiratory Tract. *Journal Of Aerosol Medicine* **1997**, *10*, 129–146.

38 Eastman, S. J., Lukason, M. J., Reeves, R., Chu, Q., Curley, J., et al., Aerosol formulations of nonviral gene transfer vectors for the treatment of cystic fibrosis. *Pediatric Pulmonology* **1999**, *234*.

39 Seville, P. C., Kellaway, I. W., Birchall, J. C., Preparation of dry powder dispersions for nonviral gene delivery by freeze-drying and spray-drying. *J. Gene Med.* **2002**, *4*, 428–437.

40 Li, H. Y., Neill, H., Innocent, R., Seville, P., Williamson, I., et al., Enhanced dispersibility and deposition of spray-dried powders for pulmonary gene therapy. *J. Drug Target.* **2003**, *11*, 425–432.

41 Mercer, T. T., Tillery, M. I., Chow, H. Y., Operating characteristics of some compressed-air nebulizers. *Am. Ind. Hyg. Assoc. J.* **1968**, *29*, 66–78.

42 Walther, W., Stein, U., Voss, C., Schmidt, T., Schleef, M., et al., Stability analysis for long-term storage of naked DNA: impact on nonviral *in vivo* gene transfer. *Anal. Biochem.* **2003**, *318*, 230–235.

43 Crook, K., McLachlan, G., Stevenson, B. J., Porteous, D. J., Plasmid DNA molecules complexed with cationic liposomes are protected from degradation by nucleases and shearing by aerosolization. *Gene Ther.* **1996**, *3*, 834–839.

44 Levy, M. S., Collins, I. J., Yim, S. S., Ward, J. M., Titchener, H. N., et al., Effect of shear on plasmid DNA in solution. *Bioprocess Engineering* **1999**, *Jan. 20*, 7–13.

45 Eastman, S. J., Tousignant, J. D., Lukason, M. J., Murray, H., Siegel, C. S., et al., Optimization of formulations and conditions for the aerosol delivery of functional cationic lipid/DNA complexes. *Hum. Gene Ther.* **1997**, *8*, 313–322.

46 Pillai, R., Petrak, K., Blezinger, P., Deshpande, D., Florack, V., et al., Ultrasonic nebulization of cationic lipid-based gene delivery systems for airway administration. *Pharm. Res.* **1998**, *15*, 1743–1747.

47 Stribling, R., Brunette, E., Liggitt, D., Gaensler, K., Debs, R., Aerosol gene delivery *in vivo*. *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 11277–11281.

48 McLachlan, G., Davidson, D. J., Stevenson, B. J., Dickinson, P., Davidson-Smith, H., et al., Evaluation *in vitro* and *in vivo* of cationic liposome-expression construct complexes for cystic fibrosis gene therapy. *Gene Ther.* **1995**, *2*, 614–622.

49 Eastman, S. J., Lukason, M. J., Tousignant, J. D., Murray, H., Lane, M. D., et al., A concentrated and stable aerosol formulation of cationic lipid/DNA complexes giving high-level gene expression in mouse lung. *Hum. Gene Ther.* **1997**, *8*, 765–773.

50 Canonico, A. E., Conary, J. T., Meyrick, B. O., Brigham, K. L., Aerosol and intravenous transfection of human alpha 1-antitrypsin gene to lungs of rabbits. *Am. J. Respir. Cell Mol. Biol.* **1994**, *10*, 24–29.

51 McDonald, R. J., Liggitt, H. D., Roche, L., Nguyen, H. T., Pearlman, R., et al., Aerosol delivery of lipid/DNA complexes to lungs of rhesus monkeys. *Pharm. Res.* **1998**, *15*, 671–679.

52 Guillaume, C., Delepine, P., Droal, C., Montier, T., Tymen, G., et al., Aerosolization of cationic lipid/DNA complexes: lipoplex characterization and optimization of aerosol delivery conditions. *Biochem. Biophys. Res. Commun.* **2001**, *286*, 464–471.

53 Densmore, C. L., Giddings, T. H., Waldrep, J. C., Kinsey, B. M., Knight, V., Gene transfer by guanidinium-cholesterol: dioleoylphosphatidyl-ethanolamine liposome-DNA complexes in aerosol. *J. Gene Med.* **1999**, *1*, 251–264.

54 Schwarz, L. A., Johnson, J. L., Black, M., Cheng, S. H., Hogan, M. E., et al., Delivery of DNA-cationic liposome complexes by small-particle aerosol. *Hum. Gene Ther.* **1996**, *7*, 731–741.

55 Raabe, O. G., Al-Bayati, M., Teague, S. V., Rasolt, A., Regional Deposition of Inhaled Monodisperse Coarse and Fine Aerosol Particles in Small Laboratory Animals. *Annals of Occupational Hygiene* **1988**, *32*, 53–63.

56 Dennis, J. H., Drug Nebuliser Design and Performance: Breath Enhanced Jet Vs. Constant Output Jet Vs. Ultrasonic. *Journal of Aerosol Medicine* **1995**, *8*, 277–280.

57 Wood, J. A., Wilson, R. S., Bray, C., Changes in salbutamol concentration in the reservoir solution of a jet nebulizer. *Br. J. Dis. Chest* **1986**, *80*, 164–169.

58 Scheule, R. K., St George, J. A., Bagley, R. G., Marshall, J., Kaplan, J. M., et al., Basis of pulmonary toxicity associated with cationic lipid-mediated gene transfer to the mammalian lung. *Hum. Gene Ther.* **1997**, *8*, 689–707.

59 Lukason, M. J., Eastman, S. J., Murray, H., Tousignant, J. D., Chu, Q., et al., *Respiratory Drug Delivery V* **1996**, 368–369.

60 Ruiz, F. E., Clancy, J. P., Perricone, M. A., Bebok, Z., Hong, J. S., et al., A clinical inflammatory syndrome attributable to aerosolized lipid-DNA administration in cystic fibrosis. *Hum. Gene Ther.* **2001**, *12*, 751–761.

61 Chadwick, S. L., Kingston, H. D., Stern, M., Cook, R. M., O'Connor, B. J., et al., Safety of a single aerosol administration of escalating doses of the cationic lipid GL-67/DOPE/DMPE-PEG5000 formulation to the lungs of normal volunteers. *Gene Therapy* **1997**, *4*, 937–942.

62 Yew, N. S., Zhao, H., Wu, I. H., Song, A., Tousignant, J. D., et al., Reduced inflammatory response to plasmid DNA vectors by elimination and inhibition of immunostimulatory CpG motifs. *Mol. Ther.* **2000**, *1*, 255–262.

63 Yew, N. S., Zhao, H., Przybylska, M., Wu, I. H., Tousignant, J. D., et al., CpG-depleted plasmid DNA vectors with enhanced safety and long-term gene expression *in vivo*. *Mol. Ther.* **2002**, *5*, 731–738.

64 Boussif, O., Lezoualc'h, F., Zanta, M. A., Mergny, M. D., Scherman, D., et al., A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethylenimine. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 7297–7301.

65 Wiseman, J. W., Goddard, C. A., McLelland, D., Colledge, W. H., A comparison of linear and branched polyethylenimine (PEI) with DCChol/DOPE liposomes for gene delivery to epithelial cells *in vitro* and *in vivo*. *Gene Ther.* **2003**, *10*, 1654–1662.

66 Rudolph, C., Lausier, J., Naundorf, S., Muller, R. H., Rosenecker, J., *In vivo* gene delivery to the lung using polyethylenimine and fractured polyamidoamine dendrimers. *J. Gene Med.* **2000**, *2*, 269–278.

67 Densmore, C. L., Orson, F. M., Xu, B., Kinsey, B. M., Waldrep, J. C., et al., Aerosol delivery of robust polyethyleneimine-DNA complexes for gene therapy and genetic immunization. *Mol. Ther.* **2000**, *1*, 180–188.

68 Gautam, A., Densmore, C. L., Xu, B., Waldrep, J. C., Enhanced gene expression in mouse lung after PEI/DNA aerosol delivery. *Mol. Ther.* **2000**, *2*, 63–70.

69 Gautam, A., Densmore, C. L., Golunski, E., Xu, B., Waldrep, J. C., Transgene expression in mouse airway epithelium by aerosol gene therapy with PEI/DNA complexes. *Mol. Ther.* **2001**, *3*, 551–556.

70 Gautam, A., Densmore, C. L., Waldrep, J. C., Inhibition of experimental lung metastasis by aerosol delivery of PEI-p53 complexes. *Mol. Ther.* **2000**, *2*, 318–323.

71 Gautam, A., Waldrep, J. C., Densmore, C. L., Koshkina, N., Melton, S., et al., Growth inhibition of established B16-F10 lung metastases by sequential aerosol delivery of p53 gene and 9-nitrocamptothecin. *Gene Ther.* **2002**, *9*, 353–357.

72 Jia, S. F., Worth, L. L., Densmore, C. L., Xu, B., Duan, X., et al., Aerosol gene therapy with PEI: IL-12 eradicates osteosarcoma lung metastases. *Clin. Cancer Res.* **2003**, *9*, 3462–3468.

73 Gautam, A., Densmore, C. L., Waldrep, J. C., Pulmonary cytokine responses associated with PEI/DNA aerosol gene therapy. *Gene Ther.* **2001**, *8*, 254–257.

74 Deshpande, D., Blanchard, J., Schuster, J., Fairbanks, D., Hobbs, C., et al., Deposition Profiles of Polyplex Formulations in Dogs Following Delivery Using the AERx® Pulmonary Delivery System. *Molecular Therapy* **2003**, *7*, S215.

75 McLachlan, G., Renwick, L., Tate, S., Painter, H. A., Lawton, A., et al., DNA deposition and transgene expression following whole lung aerosol delivery of gene therapy vectors in a large animal model. *Journal of Cystic Fibrosis* **2004**, *3*, S30.

76 Ernst, N., Ulrichskotter, S., Schmalix, W. A., Radler, J., Galneder, R., et al., Interaction of liposomal and polycationic transfection complexes with pulmonary surfactant. *J. Gene Med.* **1999**, *1*, 331–340.

77 Hardy, J. G., Newman, S. P., Knoch, M., Lung deposition from four nebulizers. *Respir. Med.* **1993**, *87*, 461–465.

78 Dhand, R., Nebulizers That Use a Vibrating Mesh or Plate with Multiple Apertures to Generate Aerosol, *Respiratory Care* **2002**, *47*, 1406–1416.

79 De Young, L. R., Chambers, F., Narayan, S., Wu, C., *Respiratory Drug Delivery VI* **1998**, 91–96.

80 Deshpande, D., Blanchard, J., Srinivasan, S., Fairbanks, D., Fujimoto, J., et al., Aerosolization of lipoplexes using AERx Pulmonary Delivery System. *AAPS PharmSci.* **2002**, *4*, E13.

81 Hayati, I., Bailey, A. I., Tadros, T. F., Investigations into the Mechanisms of Electrohydrodynamic Spraying of Liquids. *Journal of Colloid and Interface Science* **1987**, *117*, 205–230.

82 Zimlich, W. C., Ding, J. Y., Busick, D. R., Moutvic, R. R., Placke, M. E., et al., *Respiratory Drug Delivery VII* **2000**, 241–246.

9
Hydrodynamic Gene Delivery

John W. Fabre

9.1
Definition

Hydrodynamic gene delivery is the specialist term coined to describe gene delivery by the rapid infusion of large volumes of "naked" DNA into blood vessels.

"Rapid" and "large" are relative terms, which need to be defined for particular contexts. However, the critical dependence of gene delivery on the use of particular volumes and the achievement of particular flow rates, and the marked drop in gene delivery efficiency with even small decreases in either volume or flow rate are the basis for the description of the technique as "hydrodynamic".

"Naked" DNA of course refers to solutions of DNA in saline or other physiological solutions, without the addition of any vector systems for complexing the DNA.

9.2
Initial Discovery of the Technique

Given the rapidity with which intravenously injected DNA is degraded, it is at first sight surprising that hydrodynamic gene delivery is effective at all. However, the rapidly injected volume of DNA solution very probably proceeds into the circulation more or less as a bolus, mixing with blood much less than would be the case with a low-volume, low-speed intravenous injection. Moreover, the DNA that is effective in gene transfer enters the cytosol of target cells within a few minutes of delivery [1] and thus has little exposure to serum DNAases.

The first experiments using the hydrodynamic approach were reported in 1996/1997 by J. A. Wolff's group, and were aimed at gene delivery to the liver. The experiments involved DNA administration via the portal vein in mice [2] and subsequently via the portal vein and hepatic veins in rats and mice [3]. In both studies it was important to obstruct outflow of the DNA solution during the DNA infusion to obtain high levels of gene delivery (~5% of hepatocytes). DNA was administered in hypertonic solutions (15% mannitol in 0.15 M NaCl). In the 6-week-

old mice, 1 mL was administered over 30 seconds, while in the rats 15 mL was administered over 1 or 3 minutes. These are relatively large volumes (~50–60 mL · kg^{-1}), equivalent to ~4–4.5 liters when extrapolated to humans. Interestingly, portal vein delivery was equally effective in mice and rats, but retrograde delivery via hepatic veins was much less effective in rats (~1% the levels of gene delivery in mice) [3].

9.3 The Systemic Hydrodynamic Approach

In 1999, Wolff's group [4] and that of D. Liu [5] both reported that the simple injection of DNA into the tail veins of mice resulted in amazingly efficient gene delivery to the liver. This required the injection of a volume equivalent to ~10% of the body weight (~2.5 mL) over ~5 seconds, and resulted in gene delivery to ~40% of hepatocytes. The principal advantage of this approach is technical simplicity. No complex and time-consuming surgery is required.

After systemic hydrodynamic gene delivery, transgene expression can be detected in many organs, but it is overwhelmingly in the liver that gene delivery occurs. This is probably a consequence of several factors. One important factor almost certainly is that the liver has a low-pressure portal circulation, with ~80% of its blood supply coming from the portal vein. Any elevation of systemic venous pressure will therefore result in retrograde flow preferentially through the liver, and thus preferential exposure of the liver to the injected DNA. The second critical factor is probably that the capillary system in the liver (the sinusoids) is remarkably leaky. There are numerous 100–150 nm holes in the endothelial cells, known as fenestrae, through which substances can easily pass out of the circulation. Probably as important is the fact that the hepatic sinusoids do not have a basement membrane, so there is unprecedented access of the DNA solution to the tissue fluids directly bathing the hepatocytes.

Maruyama et al. [6] subsequently established systemic hydrodynamic gene delivery in the rat model. Using 8-week-old male rats (probably ~200 g body weight) they found that the injection of 25 mL (~125 mL · kg^{-1}) over 15 seconds into the tail vein gave optimal results, amounting to perhaps ~2–5% of hepatocytes. Doubling of the injection time to 30 seconds resulted in a ~50-fold reduction in gene delivery, while reducing the volume of DNA solution from 25 mL to 20 mL and 15 mL reduced gene expression ~10-fold and ~100-fold, respectively. As in the mouse studies, some gene expression (although 10–100 times lower than in liver) was seen in heart, lung, and kidney. Maruyama et al. diluted the DNA in Ringer's solution (i.e., they did not use hypertonic solutions). It is interesting to note that the level of gene delivery in the liver reported in this rat study is somewhat lower than those usually reported in mice. This is consistent with the earlier observation that retrograde gene delivery via the hepatic veins was more effective in mice [3].

The widespread expression of the transgene outside the liver, albeit at low levels, is a disadvantage from the point of view of clinical application. It also complicates

the interpretation of some experimental studies, although the liver is usually uncritically assumed to be the sole source of gene expression. However, the overwhelming problem with the systemic hydrodynamic approach is the cardiovascular risks associated with acute volume overload. Extrapolating from the rodent studies on a weight basis, the volumes that would be required in human are ~7–9 liters. The rapid intravenous infusion of such volumes is plainly out of the question in clinical practice.

9.4
The Regional Hydrodynamic Approach to the Liver

A recent development has been regional hydrodynamic gene delivery to the liver in the rat model [7]. The major objective was to solve the problems posed by the huge volumes required both for systemic hydrodynamic gene delivery (~100–125 $mL \cdot kg^{-1}$ in rodents) and for portal vein hydrodynamic gene delivery (~60 $mL \cdot kg^{-1}$ in rodents). The idea was to target *individual lobes* of the liver, and thereby achieve the critical pressure/flow conditions with physiological volumes of fluid. In these studies, 1.5 mL per 100 g of body weight (15 $mL \cdot kg^{-1}$) was delivered via a branch of the portal vein to the right lateral lobe of the liver, which accounts for ~20% of the liver mass. This volume is equivalent to ~1 liter when extrapolated to man. This approach resulted in a scattering of positive hepatocytes, similar to that reported by Maruyama et al. [6]. Outflow obstruction (achieved by placing ties on the inferior vena cava above and below the points of drainage of the hepatic veins) was crucial. The hydrodynamics were also crucial: whereas the delivery to ~200 g rats of 2 mL at 24 $mL \cdot min^{-1}$ gave little gene expression, 3 mL at the same rate was highly effective.

9.5
Gene Delivery to the Liver in Large Animals

In the report by Zhang et al. [3], six dogs were studied. However, five received DNA through the bile duct, and only one through the vasculature. In the latter case, 60 $mL \cdot kg^{-1}$ of DNA solution was delivered retrograde through the hepatic veins at 120 $mL \cdot min^{-1}$, without clamping of the portal vein to obstruct outflow of the DNA solution. The level of DNA delivery was extremely low (53 ng of luciferase per 430 g liver), which is about 100 000 times lower than was achievable in the same study with the best protocols in rodents. In the rodent experiments, however, outflow obstruction of the DNA solutions was used.

More recently, Eastman et al. [8] used balloon catheters in a rabbit model, without opening the abdomen, to mimic likely approaches in the clinic. They evaluated mainly retrograde DNA delivery either through individual hepatic veins, or through an isolated segment of the inferior vena cava to the whole liver. In neither case was the portal vein clamped to obstruct outflow of the DNA solution. DNA was delivered in a hypertonic solution of 15% mannitol, 0.15 M NaCI. The levels of gene expression

(measured as a soluble reporter gene product in serum) were < 1% of those obtained in concurrent studies using systemic hydrodynamic gene delivery in the mouse. Percutaneous catheterization of the portal vein was technically difficult in the rabbit, so DNA delivery via the portal vein was not effectively evaluated.

Hydrodynamic gene delivery targeted at the liver thus remains essentially unevaluated at this stage in large animal models.

9.6 Hydrodynamic Gene Delivery to Tissues other than Liver

Much of the work on hydrodynamic gene delivery is concerned with the liver, mainly because (for physiological reasons) the liver is the main target of the technically simple systemic hydrodynamic approach. However, the hydrodynamic approach is in principle applicable to other organs, by the delivery of DNA solutions through afferent or efferent blood vessels. For organs and tissues other than the liver, there are two potential problems. Firstly, the vascular beds of other organs are not as leaky as in liver. Secondly, retrograde delivery through the venous system might be complicated by the presence of directional valves, which are found in most (but not all) veins.

Detailed studies have been reported for skeletal muscle and kidney and are discussed below. The results in muscle have been excellent, while the levels of gene delivery in kidney were low. Outline techniques for hydrodynamic gene delivery to the gut and gonads in rodents have been reported, but no detailed results were given [9]. Our own work on retrograde hydrodynamic gene delivery to the kidney, small intestine and the adrenal gland of the rat have given only low levels of gene expression (unpublished data). It might be the case that liver and skeletal muscle are unusually favored tissues for hydrodynamic gene delivery

9.6.1 Skeletal Muscle

It is well known that simple intramuscular injection of DNA plasmids can effectively transfect the skeletal muscle cells in the region of the injection. This suggests that the skeletal muscle cells might have a particular propensity for uptake of DNA, and this is being exploited for DNA vaccination (see also Chapters 1 and 6). However, intramuscular injections are of little value for more conventionally defined gene therapy of skeletal muscle disorders. Clearly, widespread gene correction is crucial for a clinically beneficial effect in genetic disorders of skeletal muscle.

Hydrodynamic gene delivery to limb muscles was explored first in a rat model [10] and then in primates [11], again by Wolff's group. In both studies, DNA was delivered through the arterial system under conditions of outflow obstruction. In rats, 9.5 mL of DNA solution (a relatively large volume) was injected in 0.15 M NaCl over 10 seconds through the external iliac artery to the hind limbs of young (~150 g) animals. Reducing the injection volume to ~4 mL gave ~100-fold less gene

expression, while lengthening the injection time to 30 seconds virtually abolished gene expression. Interestingly, a 10 minute period of limb ischaemia prior to gene transfer increased gene expression three- to fourfold. In the primate studies, five minutes after an injection of papaverine for vasodilatation, 120 mL and 190 mL were delivered to the arms and legs, respectively, of ~10 kg macaque monkeys, over a period of 30 to 45 seconds.

This gave good results in both rats and primates. On average, ~7% of muscle cells were positive, with a range from ~1% to ~30%, depending on the muscle group. It is interesting that the vasculature of skeletal muscle has "normal" vascular endothelial cells (i.e., without fenestrae and with a basement membrane). Neither of these anatomical barriers was sufficient to prevent hydrodynamic gene delivery under the conditions used.

A less invasive technique has recently been described, using the saphenous vein for hydrodynamic gene delivery to skeletal muscles of the hind limb in rats [12]. Here, a cuff was used to isolate the limb, placed downstream of the point where the saphenous vein drains into the femoral vein. In this way, the DNA solution travels in the normal direction of flow in the saphenous vein, and then in a retrograde direction down the femoral vein to the leg muscles. Clearly, the presence of valves in the veins is not a bar to flow, at least under the conditions used for hydrodynamic delivery. Optimal conditions involved 3 mL of DNA solution injected at 10 mL · min^{-1}, which resulted in transfection rates of 3–45% in different muscle groups. Reducing the volume to 1 mL did not make a significant difference. The lower volume requirements in this study as compared to Budker et al. [10] might be a consequence of the use of the retrograde venous approach, but more probably reflects more effective outflow obstruction.

9.6.2 Kidney

Maruyama et al. [13] have performed a detailed analysis of hydrodynamic gene delivery through the renal vein to the left kidney of the rat. They report occasional positive cells in the interstitial tissues of the kidney, many fewer than are seen with hydrodynamic gene delivery to the rat liver. By careful ultrastructural analysis, they show that the positive cells are interstitial fibroblasts, in close proximity to the peritubular capillary endothelium. Neither endothelial cells nor tubular epithelial cells were ever transfected. Optimal perfusion characteristics involved the injection of 1 mL of DNA solution in 5 seconds. Reducing the volume to 0.5 mL abolished gene delivery. However, prolonging the time of injection to 60 seconds resulted in only a ~50% fall in gene expression. No gene delivery was seen outside the left kidney, using PCR techniques.

9.7 Mechanisms of Gene Delivery

The high pressure/flow in hydrodynamic gene delivery is presumably the force responsible for the extravasation of DNA into the tissue fluids. In the liver, the main route of extravasation is assumed to be the fenestrae in the sinusoidal endothelial cells. Once through the fenestrae, the DNA has direct access to hepatocytes, without intervening basement membranes, either on the endothelial cells or the hepatocytes. It was originally suggested that hepatocytes take up the DNA by receptor-mediated endocytosis [14]. This route of entry, however, is highly inefficient unless steps are taken to promote escape of the DNA from endocytic vesicles. The failure of systemic chloroquine to enhance hydrodynamic gene delivery to the liver in the rat [7] was not consistent with this hypothesis. More recently, data consistent with transient membrane disruption (driven by the high pressure/flow) have been reported [15].

In the case of skeletal muscle, the route taken by the DNA out of the circulation is a matter for conjecture, but presumably involves passage between endothelial cells in the capillary bed. The DNA must then traverse the basement membrane by diffusion. Once in the tissue fluids, cellular uptake by skeletal muscle cells might involve endocytic mechanisms [16] or transient membrane disruption.

It is interesting that complexing the DNA with cationic liposomes ([17], see also Chapter 5) or $(Lys)_{16}$-containing peptides [7] totally abolishes hydrodynamic gene delivery. The $(Lys)_{16}$ peptide/DNA nanoparticles [7] were formed under conditions where the particles were < 100 nm in diameter, and therefore able to traverse the fenestrae of the hepatic sinusoids. It was originally anticipated that condensing the DNA into nanoparticles of this size might improve hydrodynamic gene delivery. However, it seems that the momentary disruption of the plasma membrane of the hepatocyte permits access to individual DNA plasmids more readily than to nanoparticles with relatively poor diffusibility.

9.8 Safety and Clinical Applicability

Systemic hydrodynamic gene delivery has been associated with cardiac arrhythmias in mice [15] and reduced arterial blood pressure in rats [18]. In the study by Inoue et al. [18], three out of five rats given 10% of their body weight in 12 seconds via the penile vein died of respiratory failure. However, virtually all rodents survive the procedure. These problems are in any case not of direct clinical relevance, as the systemic hydrodynamic approach is not one that will be applied in the clinic.

Transient, mild elevation of hepatic and muscle enzyme levels in blood is routinely reported after hydrodynamic gene delivery, demonstrating that mild damage to these cells occurs as a consequence of the hydrodynamic procedure. Inoue et al. [18] showed that systemic hydrodynamic gene delivery in the rat, followed by the use of the rat as a liver donor in organ transplantation studies, always resulted in

recipient death. Clearly, the damage involved in hydrodynamic delivery, when added to that of the transplantation procedure (mainly transient ischaemia), was such that the transplanted liver could not sustain life in the new recipient. However, 48 hours after systemic hydrodynamic delivery to prospective liver donors, liver transplantation could be successfully performed. Zhang et al. [11] reported intimal hyperplasia in the arteries used for gene delivery to muscle in about half of the monkeys evaluated.

Rapid, high-volume infusions into blood vessels with obstructed outflow will always carry a risk. In the course of many experiments (involving > 200 rats) with regional hydrodynamic DNA delivery to lobes 2 and 3 of the liver in the rat, we have once seen necrosis of part of lobe 3. In functional terms this would not be a problem, as the liver has substantial reserve capacity and quickly regenerates. With hydrodynamic gene delivery to muscle, a circumscribed area of muscle necrosis was reported [11], which potentially has motor complications and also renal complications from myoglobinurea. Such rare complications, while not a problem in the laboratory, would be a major issue if they were to occur in the clinic.

References

1 Andrianaivo, F., Lecocq, M., Wattiaux-De Coninck, S., Wattiaux, R., Jadot, M., Hydrodynamics-based transfection of the liver: entrance into hepatocytes of DNA that causes expression takes place very early after injection. *J. Gene Med.* **2004**, *6*, 877–883.

2 Budker, V., Zhang, G., Knechtle, S., Wolff, J. A., Naked DNA delivered intraportally expresses efficiently in hepatocytes. *Gene Ther.* **1996**, *3*, 593–598.

3 Zhang, G., Vargo, D., Budker, V., Armstrong, N., Knechtle, S., Wolff, J. A., Expression of naked plasmid DNA injected into the afferent and efferent vessels of rodent and dog livers. *Hum. Gene Ther.* **1997**, *8*, 1763–1772.

4 Zhang, G., Budker, V., Wolff, J. A., High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. *Hum. Gene Ther.* **1999**, *10*, 1735–1737.

5 Liu, F., Song, Y., Liu, D., Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther.* **1999**, *6*, 1258–1266.

6 Maruyama, H., Higuchi, N., Nishikawa, Y., Kameda, S., Iino, N., Kazama, J. J., Takahashi, N., Sugawa, M., Hanawa, H., Tada, N., Miyazaki, J., Gejyo, F., High-level expression of naked DNA delivered to rat liver via tail vein injection. *J. Gene Med.* **2002**, *4*, 333–341.

7 Zhang, X., Dong, X., Sawyer, G. J., Collins, L., Fabre, J. W., Regional hydrodynamic gene delivery to the rat liver with physiological volumes of DNA solution. *J. Gene Med.* **2004**, *6*, 693–703.

8 Eastman, S. J., Baskin, K. M., Hodges, B. L., Chu, Q., Gates, A., Dreusicke, R., Anderson, S., Scheule, R. K., Development of catheter-based procedures for transducing the isolated rabbit liver with plasmid DNA. *Hum. Gene Ther.* **2002**, *13*, 2065–2077.

9 Zhang, G., Budker, V., Williams, P., Hanson, K., Wolff, J. A., Surgical procedures for intravascular delivery of plasmid DNA to organs. *Methods Enzymol.* **2002**, *346*, 125–133.

10 Budker, V., Zhang, G., Danko, I., Williams, P., Wolff, J., The efficient expression of intravascularly delivered DNA in rat muscle. *Gene Ther.* **1998**, *5*, 272–276.

11 Zhang, G., Budker, V., Williams, P., Subbotin, V., Wolff, J. A., Efficient expression of naked DNA delivered intraarterially to limb muscles of nonhuman primates. *Hum. Gene Ther.* **2001**, *12*, 427–438.

12 Hagstrom, J. E., Hegge, J., Zhang, G., Noble, M., Budker, V., Lewis, D. L., Herweijer, H., Wolff, J. A., A facile nonviral method for delivering genes and siRNAs to skeletal muscle of mammalian limbs. *Mol Ther.* **2004**, *10*, 386–398.

13 Maruyama, H., Higuchi, N., Nishikawa, Y., Hirahara, H., Iino, N., Kameda, S., Kawachi, H., Yaoita, E., Gejyo, F., Miyazaki, J., Kidney-targeted naked DNA transfer by retrograde renal vein injection in rats. *Hum. Gene Ther.* **2002**, *13*, 455–468.

14 Budker, V., Budker, T., Zhang, G., Subbotin, V., Loomis, A., Wolff, J. A., Hypothesis: naked plasmid DNA is taken up by cells *in vivo* by a receptor-mediated process. *J. Gene Med.* **2000**, *2*, 76–88.

15 Zhang, G., Gao, X., Song, Y. K., Vollmer, R., Stolz, D. B., Gasiorowski, J. Z., Dean, D. A., Liu, D., Hydroporation as the mechanism of hydrodynamic delivery. *Gene Ther.* **2004**, *11*, 675–682.

16 Wolff, J. A., Malone, R. W., Williams, P., Chong, W., Acsadi, G., Jani, A., Felgner, P. L., Direct gene transfer into mouse muscle *in vivo*. *Science* **1990**, *247*, 1465–1468.

17 Rossmanith, W., Chabicovsky, M., Herkner, K., Schulte-Hermann, R., Cellular gene dose and kinetics of gene expression in mouse livers transfected by high-volume tail-vein injection of naked DNA. *DNA Cell Biol.* **2002**, *21*, 847–853.

18 Inoue, S., Hakamata, Y., Kaneko, M., Kobayashi, E., Gene therapy for organ grafts using rapid injection of naked DNA: application to the rat liver. *Transplantation* **2004**, *77*, 997–1003.

10
DNA Pharmaceuticals for Skin Diseases

Vitali Alexeev and Jouni Uitto

10.1
Introduction

The ability to manipulate nucleic acids by the means of sequencing, synthesis, and *in vitro* transcription/translation, as well as by amplification of short and long DNA fragments by the polymerase chain reaction, has allowed us to extend our understanding of many human diseases at the molecular level. This knowledge has led to attempts to invent new nucleic acid-based therapeutics. These efforts have catalyzed the implementation of innovative strategies, and such approaches are at the cutting edge of the new molecular medicine that is predicted to transform traditional medicine profoundly in the not too distant future.

At present, the nucleic acid-based approaches employed by molecular medicine can be divided into two broad categories: those that rely on recombinant DNA molecules and those that utilize synthetic DNA. The former approaches are based on utilization of traditional experimental techniques that enable individual genes and DNA sequences to be manipulated, while the latter are based on technological innovations that have allowed creation of DNA or RNA molecules from single nucleotides, as well as on the intrinsic property of the nucleic acids to interact with each other thanks to the principle of complementarity.

During the past few decades, recombinant DNA technology has largely substituted the conventional methods of production of proteins, processed from human or animal sera or tissues. This progress has allowed large-scale manufacturing of various pharmaceutical products, including drugs that could not be produced by conventional methods. Based on the rapidly expanding knowledge of the mutation database in human diseases, attempts have also been made to use recombinant DNA for treatment of a number of genetic disorders. Such attempts to replace defective, disease-causing genes with copies of their recombinant wild-type counterparts have formed the backbone of the gene therapy field [1, 2]. Subsequently, another biomedical application of recombinant DNA technology was based on early observations that intramuscular injection of a plasmid DNA encoding the bacterial protein β-galactosidase resulted in the expression of the protein *in vivo* [3]. These

experiments, developed on in the 1990s and during the early part of this century, have supported the notion of DNA vaccination (see Chapters 1 and 6).

This overview reviews the potential for application of nucleic acids in the treatment of selected diseases, highlighting dermatologic conditions as paradigms of diseases in which recent progress has been made both in molecular genetics and in DNA pharmaceuticals.

10.2 Recombinant DNA-Based Skin Gene Therapy

10.2.1 Correction of Genetic Disorders

Early development in recombinant DNA technology put forward the idea that molecular drugs could be developed to cure inherited diseases by transfer of recombinant genetic material into patients' cells. This seemingly straightforward concept of direct application of recombinant DNA technology has developed into a recognized field in the biomedical sciences: gene therapy medicine. Two general principles can be utilized to correct the mutations: either gene targeting and repair or gene replacement. The former approach employs oligonucleotides or short DNA fragments capable of binding and interacting with homologous loci [4, 5], while the latter employs recombinant, wild-type copies of the coding sequences of the defective genes [6]. Introduction of a functional copy (or copies) of the gene rendered defective by the mutations could be accomplished either by physical delivery or by virus-mediated gene transfer [7]. While the viral approaches have afforded extremely efficient delivery of the recombinant DNA into the cells, the safety concerns surrounding the use of viruses on human subjects [8] have made nonviral gene therapy approaches an attractive alternative [9].

The accessibility and ease of inspection of skin and the potential to correct genetic mutations either *in vivo* or *ex vivo* make heritable skin disorders attractive candidate diseases for gene therapy [10–12]. In the *in vivo* approach, genetic material, such as recombinant wild-type copies of the defective gene, is directly introduced into the skin by injection (see also Chapter 7), electroporation (see also Chapters 11 and 12), or other physical delivery methods (Figure 10.1). In contrast, the *ex vivo* delivery approach could involve removal of a small skin specimen from the patient, followed by propagation of skin cells such as epidermal keratinocytes or dermal fibroblasts in culture. Upon the introduction of genetic material into the cultured cells, the genetically altered cells can be cultured to form a skin graft, which can be applied back to the patient (Figure 10.1). In this context, it should be noted that many genodermatoses are generalized disorders and the clinical manifestation can affect the entire skin. This situation currently presents an obvious problem, as most of the strategies developed so far are applicable for treatment of only limited areas of skin. Nevertheless, local correction of the disease phenotype in a limited area of skin may be beneficial for some patients. As an example, treatment of the hands in

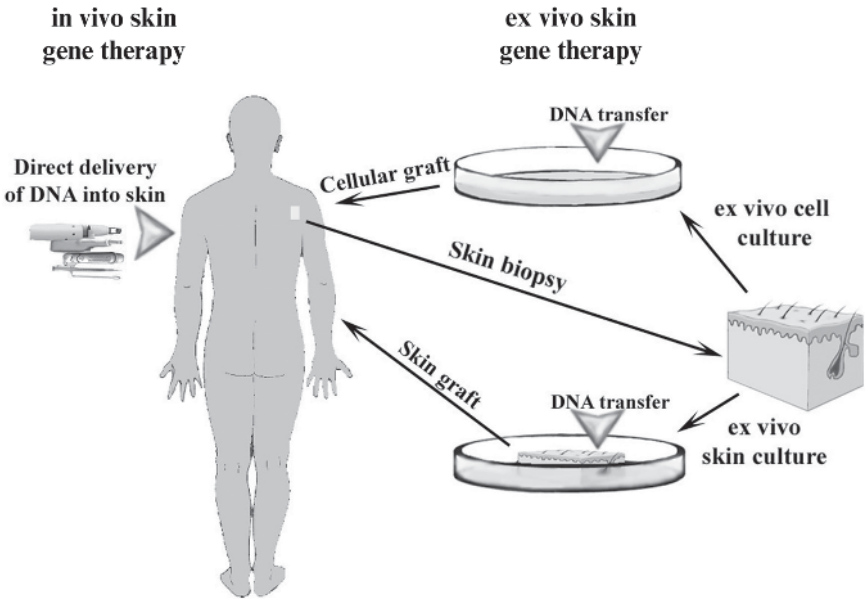

Figure 10.1 Principles of the two primary strategies for cutaneous gene therapy. In the case of the *in vivo* strategy, the genes are delivered directly into the skin. In contrast, in the *ex vivo* strategy, a skin biopsy is removed and genetic material is introduced into cultured cells (keratinocytes or fibroblasts) or skin explants. The transduced cells, tissues, or human skin reconstructs are then grafted back to the original donor.

a patient with a severe form of recessive dystrophic epidermolysis bullosa (the Hallopeau–Siemens type), which manifests as generalized blistering and scarring but particularly affects the hands by development of pseudosyndactyly (fusion of the digits to form a mitten hand), would be expected to result in substantial improvement in manual dexterity and overall quality of life of the affected individual.

The recent discovery and characterization of the epidermal stem cells, which represent the basis for continuous regeneration of the epidermis [13–15], also make an *ex vivo* approach to gene therapy possible. Specifically, genetically modified stem cells, once grafted back into the patient's skin, could continuously repopulate the epidermis and produce a longlasting therapeutic effect. Unfortunately, knowledge of epidermal stem cell characteristics is currently limited, although further characterization of these cells through joint efforts of dermatologists, geneticists, and cell biologists should result in acquisition of knowledge that will allow genetic manipulation of the epidermal cells and make cutaneous gene therapy practically applicable in the near future.

10.2.2
"Suicide" Gene Therapy

Development of recombinant DNA technology and gene delivery systems has resulted in another promising concept: "suicide" gene therapy [16, 17]. In this approach, the "suicide" gene, such as the herpes simplex thymidine kinase (HSV-TK) gene, is introduced into tumor cells under the control of a tumor specific-promoter that restricts the expression of the transgene to the tumor cells. Thus, only cells expressing HSV-TK are able to convert the prodrug ganciclovir into a highly toxic derivative, which then disrupts DNA replication and results in death of the tumor cells. Without HSV-TK, ganciclovir is virtually nontoxic for cells, and so is not harmful for cells in normal tissues, thus restricting the "suicide" effects only to the HSV-TK-expressing tumors. This approach has been demonstrated to be effective for treatment of neuroblastoma and melanoma in preclinical animal models, and is currently in clinical trials for treatment of these conditions [18–20].

10.2.3
Genetic Pharmacology

A particularly promising application of gene therapy relates to genetic pharmacology [21]. This approach is based on the expression of vectors encoding therapeutically beneficial proteins that, upon administration into the tissues, result in the expression of the corresponding protein and in clinical improvement of the patient. This approach is amply demonstrated by recombinant production of various clotting factors for hematological disorders as well as by the use of erythropoietin to enhance red blood cell proliferation in patients with chronic anemia [22–24]. Similarly, the expression of various hormones and growth factors has been extensively studied, and these approaches are being tested in clinical trials for metabolic disorders, as well as in attempts to enhance tissue regeneration.

10.3
DNA Vaccines

In 1796, Dr. Edward Jenner invented a vaccine against smallpox, and about one hundred years later Louis Pasteur proposed the "germ theory of disease". He then discovered the power of vaccines against rabies, and vaccination has enjoyed enormous success in the improvement of public health. Vaccinations are mainly used for prevention of infectious diseases through the induction of high levels of antigen-specific neutralizing antibodies. Thanks to this progress, the majority of the infectious diseases of the 18th and 19th centuries are now an occasional and relatively minor problem.

The advent of newly emerging pathogens (HIV, SARS, newly discovered pneumonia-causing bacteria, etc.), as well as the development of immunological methods targeting cancer, has necessitated the search for new type of vaccines usable not

only for prophylactic purposes but also for treatment of these diseases. The original idea of genetic vaccination emanated from the observations that injection of naked plasmid DNA encoding β-galactosidase resulted in transfection of muscle cells and in the expression of the protein *in vivo* [3]. In similar experimental settings, the intramuscular delivery of the plasmid DNA encoding influenza A virus protein resulted in the induction of specific humoral and cellular responses that protect against this viral challenge [25]. These dramatic findings have resulted in the development of simple and potentially powerful DNA vaccination technologies.

It was subsequently found that that the transfected muscle did not directly prime the T-cells but interacted with professional antigen-presenting cells (APCs), which "collect" antigens and only then present them to the T cells [26, 27]. The process of antigen transfer (i.e., "cross-presentation") was found to be a prerequisite and a major route by which antigens of the intracellular pathogens elicit MHC class I-dependent cytotoxic immune responses [28, 29]. A related and extremely interesting

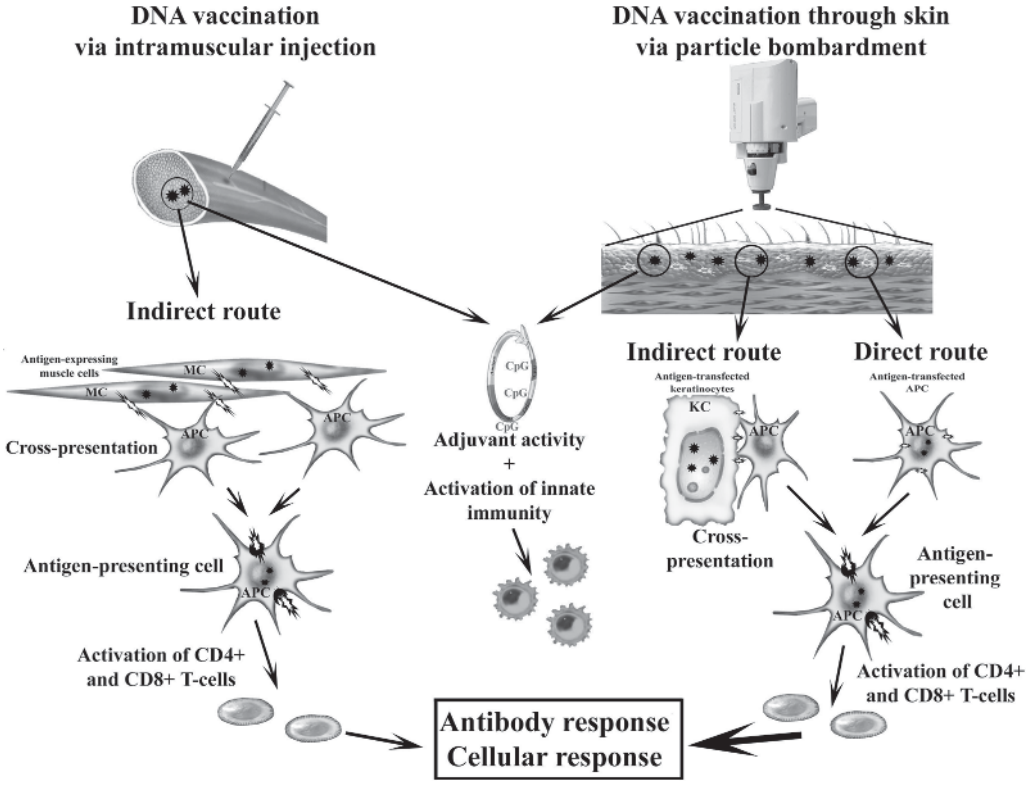

Figure 10.2 A model of DNA vaccination. After direct injection (muscles) or biolistic administration (skin) of the DNA recombinant vaccine, transduced muscle cells (MCs) or keratinocytes (KCs) express the antigen, which can be recognized by the antigen-presenting cells (APCs) (indirect route). APCs can also be transfected directly (direct route). CpG sequences in the plasmid DNA stimulate the innate immunity. The outcome of both direct and indirect routes is the activation of the innate and antigen-specific adaptive immune responses.

observation is that plasmid DNA encoding the antigen is not only the template for protein production, but also has intrinsic properties of attracting immune cells, due to the presence of immunostimulatory sequences in the bacterial DNA backbone used for plasmid construction. These sequences contain unmethylated CpG motifs found in bacterial genomes at frequencies 20 times higher than found in vertebrate DNA. These motifs represent the molecular pattern capable of activating the innate immune system and inducing secretion of cytokines that contribute to the T helper 1 (Th-1) immune responses [30, 31]. Collectively, these pioneering studies revealed that genetic vaccination can serve as a vehicle to ensure antigen production and simultaneously possess immunostimulatory, adjuvant properties.

Joint research by dermatologists and immunologists has shown that the skin not only represents the physical barrier that protects our bodies from external trauma and pathogen invasion, but is also home to a well balanced immunological complex capable of inducing active immune responses to foreign molecules or organisms. This latter property of skin, combined with its accessibility and regenerative potential, makes it an attractive organ for immunization, particularly for DNA vaccination (Figure 10.2).

10.3.1 DNA Vaccination Through Skin

The skin has evolved as a barrier to prevent the entry of pathogens into the body. The two principal layers of the skin, epidermis and dermis, serve as the first line defense against foreign pathogens, and they contain an efficient immune surveillance complex, which includes Langerhans cells, melanocytes (epidermis), and dendritic cells (dermis). There are also additional cell types that actively participate in innate immunity, such as macrophages and mast cells. Skin is also rich in lymphatic vasculature to drain body fluids, and this network provides an efficient route for trafficking of antigen-presenting cells for the purpose of presenting antigens to the T cells for initiation of adoptive immunity. Depending on the method of delivery, DNA-based vaccines can be targeted to specific locations in the skin, and in conjunction with traditional or genetic adjuvants they can elicit specific immune responses [32]. Skin immunization has so far enjoyed most success when "gene gun"-based DNA delivery systems have been employed. This approach allows effective delivery of the recombinant DNA molecules into both dermal and epidermal components. The utilization of tissue-specific promoters has also been advantageous: several, including involucrin for the expression of the transgene in the upper epidermis, keratin 14 (basal keratinocytes), collagen 1 (dermal fibroblasts), and tyrosinase (melanocytes) have been successfully used (Figure 10.3).

In contrast with intramuscular immunization, for which cross-presentation of the antigen is a prerequisite, particle bombardment by gene gun may also result in direct deposition of DNA-coated gold particles in Langerhans cells and dermal macrophages (Figure 10.2). These cells act as antigen-presenting cells, resulting in the elicitation of the Th-2 responses and predominant production of IgG1 antibodies. In addition, skin vaccination may elicit active innate immune responses, which

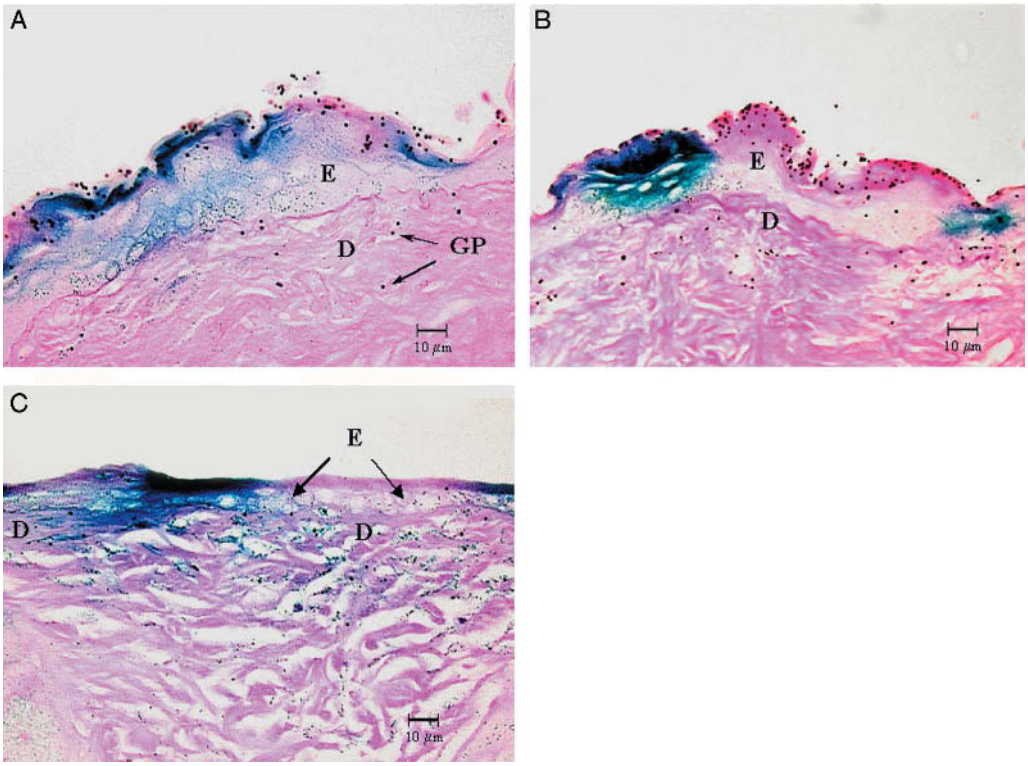

Figure 10.3 Gene gun-mediated, tissue-specific expression of the β-galactosidase in mouse skin.
Panel (A) Involucrin promoter-driven expression of the β-galactosidase (blue) in the upper epidermis. Panel (B) Keratin 14 promoter-driven expression of the β-galactosidase in the basal keratinocytes and in the progeny of the DNA-transduced epidermal stem cells. Panel (C) CMV promoter-driven expression of the β-galactosidase detected in dermis (D) and epidermis (E). Gold particles (GP) can be seen as dark dots both in epidermis and dermis.

could be advantageous for the development of DNA vaccines against various types of cancers, including skin carcinomas and melanoma.

10.3.2
DNA Vaccines Against Skin Cancers

A significant distinction between vaccination against infectious diseases and vaccination against cancer lies in the fact that the majority of cancers may not be associated with infectious agents, consisting instead of cells with the inherently weak immunogenicity of tumor antigens, which may be only slightly different from self-antigens. Ideally, the development of effective anticancer vaccines requires a tumor antigen that is highly expressed by tumor cells and not by normal cells, and preferably that such a molecule be essential for tumor cell growth and/or survival

[33]. Unfortunately, only a few tumor antigens appear to fulfill these requirements [34]. However, there are target tumor antigens suitable for DNA vaccination purposes, either produced by specialized cells and/or representing lineage-specific proteins, as seen in several epithelial cancers that express keratinocyte-specific proteins or in melanoma. Melanoma tumor antigens are predominantly products of genes overexpressed or mutated in tumor cells or they represent normal differentiation proteins expressed in a manner specific to the cell lineage. As an example, such melanocyte-specific antigens include gp100, melanoma antigens recognized by T cells 1 (MART-1), tyrosinase, and tyrosinase-related proteins 1 and 2. These proteins represent important candidates for tumor regression antigens, which may turn out to be therapeutically important targets. Vaccines for many of these antigens are currently being tested in clinical trials [35].

10.4
Physical Methods of DNA Delivery

Gene therapy and DNA vaccination utilizing recombinant DNA molecules have both advantages and limitations. Although these therapeutic strategies have in many cases advanced to clinical trials during the last decade, the major question remaining is whether the exciting results obtained in preclinical animal models will translate into efficacy in human subjects. In this context, the choice of the recombinant vector, the dose, volume, and site of delivery could be critical for the elicitation of

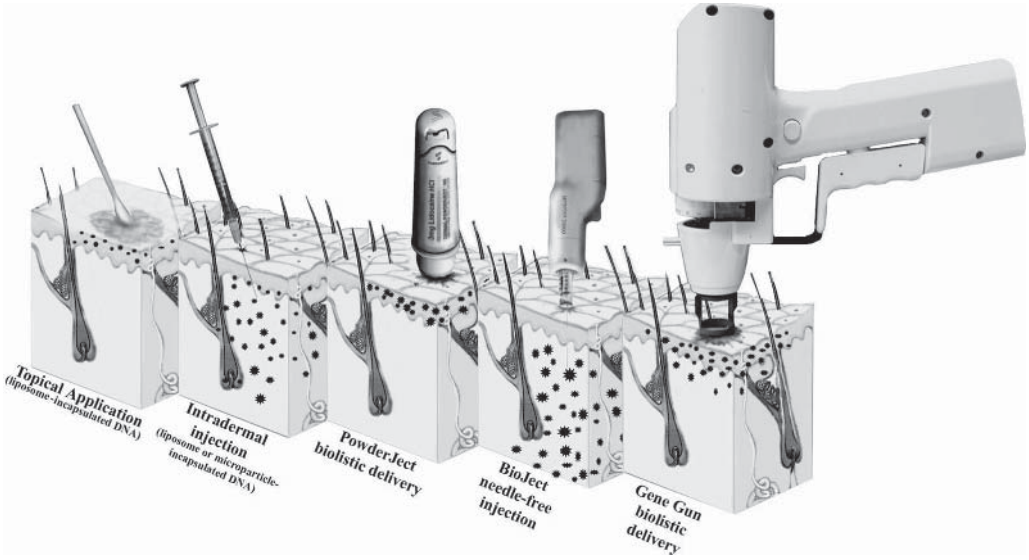

Figure 10.4 Physical methods of DNA delivery. The delivery of DNA into the skin can be mediated by direct topical application, injection, needle-free jet injection, powder immunization, or gene gun. Relative depth of penetration of the recombinant DNA into skin is represented by stars (✷).

significant results. Physical methods used for transfection of gene/antigen-encoding plasmids, such as electroporation, biolistic needle-free jet injection, powder immunization, or microparticle delivery, may overcome some of the problems and may improve the applicability and efficacy of gene therapy and DNA vaccination (Figure 10.4).

10.4.1 Delivery of DNA to the Skin by Particle Bombardment

Particle-mediated DNA delivery technologies have been developed as physical gene-transfer methods for various *in vivo*, *ex vivo*, and *in vitro* applications. The basic concept is to deliver naked DNA plasmids directly into the target cells by using microparticles as physical carriers of DNA. This technology was first established for plant gene transfer and was described as "biological ballistic" (biolistic) in 1987 by Sanford and colleagues [36]. Helium-driven gene gun systems based on this concept have been developed, and include the Accell gene gun (Agracetus, Inc.) and the Helios gene gun (Bio-Rad Laboratories).

For intracellular delivery, particle bombardment deploys a high-velocity stream of physical carrier particles, which can be coated with a number of different macromolecules, including nucleic acids, proteins, or peptides. Such coated particles can then be transferred into hundreds or even thousands of cells as a result of a single delivery application. The efficiency of the delivery is clearly affected by a number of parameters, including the size and the material of the carrier particles, the density of the particles and DNA, and the acceleration that distributes the particles to the skin or other tissues. The carrier particles for bombardment are usually made of gold, in the form of low-toxic, unreactive spheres of subcellular size (0.5–5 μm), with sufficient density to penetrate the skin. The process involves precipitation of plasmid DNA onto the gold beads, and the DNA/gold particle complexes are then coated around the inside of Teflon tubing, which can be cut into short cartridges. These cartridges are loaded into the gene gun, and the coated gold beads are accelerated from the cartridges by a high-pressure helium blast (procedures review in ref. [37]).

The gene gun delivers the majority of the gold beads into the epidermal layers (Figure 10.4). The distribution of the gold beads in the skin, as well as the damage to the treated tissue, is mainly dependant on the velocity of the particles, the thickness of the skin, and the site of bombardment. Although it has been suggested that these methods may not be suitable for cutaneous gene therapy in humans due to the potential mechanical damage, gene gun delivery of DNA to the epidermis has been successfully employed in DNA vaccine studies [38–41]. In fact, it has been reported that skin vaccination of mice with amounts of less than one microgram of DNA encoding one of the influenza virus proteins by particle bombardment was able to protect the mice from viral challenge.

Although potentially effective, delivery of DNA to skin by particle bombardment has its limitations. Firstly, the cost of delivery devices and the carrier gold particles is considerable. Secondly, the Helios gene gun (Bio-Rad Laboratories) is designated

for "research use only". Nevertheless, several similar methods and handheld devices for needle-free delivery of nucleic acids and proteins into the skin are currently being developed.

10.4.2 Microparticles for DNA Delivery

DNA vaccines are typically delivered either by intramuscular injection or by biolistic propulsion of DNA-coated beads into the epidermis by gene gun. Initial studies demonstrated that DNA vaccines lack the ability to invade cells efficiently and are highly susceptible to degradation in the nucleus. These observations prompted the development of microparticle-mediated DNA vaccine delivery systems. The rationale for the use of microparticles as delivery systems for injectable vaccines is based on their ability to be phagocytized by antigen-presenting cells, which has been demonstrated both *in vitro* and *in vivo* [42, 43]. In fact, early studies showed that microparticles in the 1–3 μm size range can be efficiently taken up by macrophages. In addition, it was shown that the surface charge and hydrophobicity characteristics of the microparticles can modify the extent of uptake [44, 45]. More recent studies have shown that novel cationic microparticle formulations with DNA absorbed onto their surfaces can be much more efficient in DNA delivery. It has also been suggested that microparticle-mediated DNA delivery in humans is relatively safe, since microparticles have been used as controlled release delivery systems in certain licensed products. Microparticle-encapsulated DNA vaccines and human papilloma virus antigen proteins have been tested in phase I and II clinical trials [46, 47]. Finally, microparticles with absorbed DNA vaccines encoding HIV antigens have recently entered human clinical trials in healthy volunteers [48, 49]. It is conceivable, therefore, that further optimization of microparticle formulation and DNA absorption may significantly improve their use as a DNA delivery system. Moreover, recent developments of new devices designed to allow the delivery of microparticle-encapsulated DNA into the skin may significantly improve the outcome of DNA vaccination.

10.4.3 Genetic Immunization by Jet Injection

The optimization of particle-mediated gene transfer and the biolistic administration of plasmid DNA by gene gun have prompted the development of new approaches for the efficient delivery of DNA (Figure 10.4). Genetic immunization by jet injection represents a combination of nanoparticle-based DNA delivery systems with commercially available needle-free jet injection immunization devices, such as the Biojector 2000. Needle-free jet injection has been investigated extensively as a method to immunize laboratory animals such as mice, rabbits, pigs, dogs, and monkeys [50–53]. Many initial studies have demonstrated that both intramuscular and intradermal jet injection of antigen-encoding DNA are 20 times more efficient than traditional needle injection in the induction of immune responses. Subsequent

investigations showed that nanoparticle-coated DNA delivered by jet injection additionally enhanced the induction of immune responses by a factor of ~200 on average. Although the majority of studies on animals have been successful, several clinical trials on humans have reported somewhat equivocal results. Nevertheless, the majority of these studies found statistical benefits in enhancing immune response by nanoparticle-mediated DNA jet injection [54–56], suggesting that further optimization of the nanoparticle formulation and jet injection devices may significantly improve the efficacy of DNA immunization.

10.4.4
Epidermal Powder Immunization

Skin immunization has recently received additional attention, with a variety of delivery technologies currently being developed. One handheld device for powder immunization, developed by PowderJect Vaccines, Inc., is based on the same general principle as the Helios gene gun. The delivery device for the powder immunization is a single-use device, composed of a helium microcylinder, a vaccine-containing cassette, a nozzle, and a silencer. The microcylinder is filled with medical grade helium gas to nominal pressure of 45 bar. The cassette is constructed of an elastomer washer with rupture membranes housing the powder vaccine. Upon activation of the device, helium is released from the gas portal, resulting in rupture of the membranes of the cassette with accelerated introduction of the vaccine powder into the viable epidermis [57]. Epidermal powder immunization (EPI) was originally developed as a needle-free immunization technology, designed to deliver powder protein vaccines to the epidermis (Figure 10.4). Preclinical studies have demonstrated that it is possible to deliver antigens directly to the antigen-presenting cells of the epidermis by EPI. Nearly all epidermal Langerhans cells at the site of EPI contain the antigen, and many antigen-containing cells are detected in the draining lymph nodes where antigen presentation normally takes place [58].

The preclinical data suggest that the efficacy of the EPI can be further improved by optimizing the helium gas pressure and the physical characteristics of the powder. Ongoing clinical trials should allow the evaluation of these parameters, but the development of epidermal powder immunization as a leading needle-free skin delivery technology has already yielded encouraging results both in animal experiments and now in initial clinical trials.

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References

1 Verma, I. M., Somia, N., Gene therapy – promises, problems and prospects. *Nature* **1997**, *389*, 239–342.
2 Mulligan, R. C., The basic science of gene therapy. *Science* **1993**, *260*, 926–932.
3 Wolff, J. A., Malone, R. W., Williams, P., et al., Direct gene transfer into mouse muscle *in vivo*. *Science* **1990**, *247*, 1465–1468.
4 Igoucheva, O., Alexeev, V., Yoon, K., Oligonucleotide-directed mutagenesis and targeted gene correction: a mechanistic point of view. *Curr. Mol. Med.* **2004**, *4*, 445–463.
5 Kunzelmann, K., Legendre, J. Y., Knoell, D. L., et al., Gene targeting of CFTR DNA in CF epithelial cells. *Gene Ther.* **1996**, *3*, 859–867.
6 Friedmann, T., The evolving concept of gene therapy. *Hum. Gene Ther.* **1990**, *1*, 175–181.
7 Verma, I. M., Naviaux, R. K., Human gene therapy. *Curr. Opin. Genet Dev.* **1991**, *1*, 54–59.
8 Jooss, K., Chirmule, N., Immunity to adenovirus and adeno-associated viral vectors: implications for gene therapy. *Gene Ther.* **2003**, *10*, 955–963.
9 Li, S., Huang, L., Nonviral gene therapy: promises and challenges. *Gene Ther.* **2000**, *7*, 31–34.
10 Choate, K. A., Khavari, P. A., Direct cutaneous gene delivery in a human genetic skin disease. *Hum Gene Ther.* **1997**, *8*, 1659–1665.
11 Dellambra, E., Vailly, J., Pellegrini, G., et al., Corrective transduction of human epidermal stem cells in laminin-5-dependent junctional epidermolysis bullosa. *Hum. Gene Ther.* **1998**, *9*, 1359–1370.
12 Ghazizadeh, S., Harrington, R., Taichman, L., *In vivo* transduction of mouse epidermis with recombinant retroviral vectors: implications for cutaneous gene therapy. *Gene Ther.* **1999**, *6*, 1267–1275.
13 Gambardella, L., Barrandon, Y., The multifaceted adult epidermal stem cell. *Curr. Opin. Cell Biol.* **2003**, *15*, 771–777.
14 Lavker, R. M., Sun, T. T., Oshima, H., et al., Hair follicle stem cells. *J. Investig. Dermatol. Symp. Proc.* **2003**, *8*, 28–38.
15 Ghazizadeh, S., Taichman, L. B., Organization of stem cells and their progeny in human epidermis. *J. Invest. Dermatol.* **2005**, *124*, 367–372.
16 Freeman, S. M., Abboud, C. N., Whartenby, K. A., et al., The "bystander effect": tumor regression when a fraction of the tumor mass is genetically modified. *Cancer Res.* **1993**, *53*, 5274–5283.
17 Freeman, S. M., Whartenby, K. A., Freeman, J. L., et al., *In situ* use of suicide genes for cancer therapy. *Semin. Oncol.* **1996**, *23*, 31–45.
18 Klatzmann, D., Cherin, P., Bensimon, G., et al., A phase I/II dose-escalation study of herpes simplex virus type 1 thymidine kinase "suicide" gene therapy for metastatic melanoma. Study Group on Gene Therapy of Metastatic Melanoma. *Hum. Gene Ther.* **1998**, *9*, 2585–2594.

19 Cohen, J. L., Boyer, O., Klatzmann, D., Suicide gene therapy of graft-versus-host disease: immune reconstitution with transplanted mature T cells. *Blood* **2001**, *98*, 2071–2076.

20 Braunberger, E., Cohen, J. L., Boyer, O., et al., T-Cell suicide gene therapy for organ transplantation: induction of long-lasting tolerance to allogeneic heart without generalized immunosuppression. *Mol. Ther.* **2000**, *2*, 596–601.

21 Greenhalgh, D. A., Rothnagel, J. A., Roop, D. R., Epidermis: an attractive target tissue for gene therapy. *J. Invest. Dermatol.* **1994**, *103*, 63S–69S.

22 Klinman, D. M., Conover, J., Leiden, J. M., et al., Safe and effective regulation of hematocrit by gene gun administration of an erythropoietin-encoding DNA plasmid. *Hum. Gene Ther.* **1999**, *10*, 659–665.

23 High, K. A., Clinical gene transfer studies for hemophilia B. *Semin. Thromb. Hemost.* **2004**, *30*, 257–267.

24 Gomez-Vargas, A., Hortelano, G., Nonviral gene therapy approaches to hemophilia. *Semin. Thromb. Hemost.* **2004**, *30*, 197–204.

25 Ulmer, J. B., Donnelly, J. J., Parker, S. E., et al., Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* **1993**, *259*, 1745–1749.

26 Doe, B., Selby, M., Barnett, S., et al., Induction of cytotoxic T lymphocytes by intramuscular immunization with plasmid DNA is facilitated by bone marrow-derived cells. *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 8578–8583.

27 Corr, M., Lee, D. J., Carson, D. A., et al., Gene vaccination with naked plasmid DNA: mechanism of CTL priming. *J. Exp. Med.* **1996**, *184*, 1555–1560.

28 Houde, M., Bertholet, S., Gagnon, E., et al., Phagosomes are competent organelles for antigen cross-presentation. *Nature* **2003**, *425*, 402–406.

29 Heath, W. R., Belz, G. T., Behrens, G. M., et al., Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens. *Immunol Rev* **2004**, *199*, 9–26.

30 Krieg, A. M., From A to Z on CpG. *Trends Immunol* **2002**, *23*, 64–65.

31 Krieg, A. M., A role for Toll in autoimmunity. *Nat Immunol* **2002**, *3*, 423–424.

32 Peachman, K. K., Rao, M., Alving, C. R., Immunization with DNA through the skin. *Methods* **2003**, *31*, 232–242.

33 Schultze, J. L., Maecker, B., von Bergwelt-Baildon, M. S., et al., Tumour immunotherapy: new tools, new treatment modalities and new T-cell antigens. *Vox Sang.* **2001**, *80*, 81–89.

34 Stevenson, F. K., Rice, J., Zhu, D., Tumor vaccines. *Adv. Immunol.* **2004**, *82*, 49–103.

35 Talebi, T., Weber, J. S., Peptide vaccine trials for melanoma: preclinical background and clinical results. *Semin. Cancer Biol.* **2003**, *13*, 431–438.

36 Klein, R. M., Wolf, E. D., Wu, R., et al., High-velocity microprojectiles for delivering nucleic acids into living cells. *Biotechnology* **1992**, *24*, 384–386.

37 Wang, S., Joshi, S., Lu, S., Delivery of DNA to skin by particle bombardment. In: Heiser, W. C. (Ed.), *Methods Mol. Biol.* Totowa, NJ, USA: Humana Press **2004**, *245*, 185–196.

38 Lunn, D. P., Soboll, G., Schram, B. R., et al., Antibody responses to DNA vaccination of horses using the influenza virus hemagglutinin gene. *Vaccine* **1999**, *17*, 2245–2258.

39 Macklin, M. D., McCabe, D., McGregor, M. W., et al., Immunization of pigs with a particle-mediated DNA vaccine to influenza A virus protects against challenge with homologous virus. *J. Virol.* **1998**, *72*, 1491–1496.

40 Haynes, J. R., McCabe, D. E., Swain, W. F., et al., Particle-mediated nucleic acid immunization. *J. Biotechnol.* **1996**, *44*, 37–42.

41 Swain, W. F., Macklin, M. D., Neumann, G., et al., Manipulation of immune responses via particle-mediated polynucleotide vaccines. *Behring Inst. Mitt.* **1997**, 73–78.

42 Tabata, Y., Ikada, Y., Macrophage activation for antitumour function by muramyl dipeptide-protein conjugates. *J. Pharm. Pharmacol.* **1990**, *42*, 13–19.

43 Tabata, Y., Uno, K., Yamaoka, T., et al., Effects of recombinant alpha-interferon-gelatin conjugate on *in vivo* murine tumor cell growth. *Cancer Res.* **1991**, *51*, 5532–5538.

44 Andrianov, A. K., Payne, L. G., Polymeric carriers for oral uptake of microparticulates. *Adv. Drug Deliv. Rev.* **1998**, *34*, 155–170.

45 Evans, R. K., Zhu, D. M., Casimiro, D. R., et al., Characterization and biological evaluation of a microparticle adjuvant formulation for plasmid DNA vaccines. *J. Pharm. Sci.* **2004**, *93*, 1924–1939.

46 Dale, C. J., Liu, X. S., De Rose, R., et al., Chimeric human papilloma virus-simian/human immunodeficiency virus virus-like-particle vaccines: immunogenicity and protective efficacy in macaques. *Virology* **2002**, *301*, 176–187.

47 Liu, M., Acres, B., Balloul, J. M., et al., Gene-based vaccines and immunotherapeutics. *Proc. Natl. Acad. Sci. USA* **2004**, 101 Suppl. 2, 14567–14571.

48 Park, J., Lee, S. H., Kim, A., Microparticle and liquid formulation of a novel HIV protease inhibitor. *Pharm. Dev. Technol.* **2002**, *7*, 297–303.

49 Jiang, W., Gupta, R. K., Deshpande, M. C., et al., Biodegradable poly(lactic-co-glycolic acid) microparticles for injectable delivery of vaccine antigens. *Adv. Drug Deliv. Rev.* **2005**, *57*, 391–410.

50 Haensler, J., Verdelet, C., Sanchez, V., et al., Intradermal DNA immunization by using jet-injectors in mice and monkeys. *Vaccine* **1999**, *17*, 628–638.

51 Ren, S., Li, M., Smith, J. M., et al., Low-volume jet injection for intradermal immunization in rabbits. *BMC Biotechnol.* **2002**, *2*, 10.

52 Anwer, K., Earle, K. A., Shi, M., et al., Synergistic effect of formulated plasmid and needle-free injection for genetic vaccines. *Pharm. Res.* **1999**, *16*, 889–895.

53 Gramzinski, R. A., Millan, C. L., Obaldia, N., et al., Immune response to a hepatitis B DNA vaccine in Aotus monkeys: a comparison of vaccine formulation, route, and method of administration. *Mol. Med.* **1998**, *4*, 109–118.

54 Parent du Chatelet, I., Lang, J., Schlumberger, M., et al., Clinical immunogenicity and tolerance studies of liquid vaccines delivered by jet-injector and a new single-use cartridge (Imule): comparison with standard syringe injection. Imule Investigators Group. *Vaccine* **1997**, *15*, 449–458.

55 Williams, J., Fox-Leyva, L., Christensen, C., et al., Hepatitis A vaccine administration: comparison between jet-injector and needle injection. *Vaccine* **2000**, *18*, 1939–1943.

56 Jackson, L. A., Austin, G., Chen, R. T., et al., Safety and immunogenicity of varying dosages of trivalent inactivated influenza vaccine administered by needle-free jet injectors. *Vaccine* **2001**, *19*, 4703–4709.

57 Chen., D., Burger, M., Chu, Q., et al., Epidermal powder immunization: cellular and molecular mechanisms for enhancing vaccine immunogenicity. *Virus Res.* **2004**, *103*, 147–153.

58 Dean, H. J., Chen, D., Epidermal powder immunization against influenza. *Vaccine* **2004**, *23*, 681–686.

11
Electrotransfection – An Overview

Capucine Trollet, Pascal Bigey, and Daniel Scherman

Efficient and safe delivery of DNA *in vivo* is a requirement for several purposes, such as study of gene function or gene therapy applications. In particular, the sequencing of the human genome will inequitably result in the cloning and the functional characterization of numerous new proteins, which will require both *in vitro* and *in vivo* functional studies. Although the problem of *in vitro* gene transfer is reasonably solved by means of cationic lipid or polymer transfection, calcium phosphate precipitation, or electroporation, it is much more difficult to achieve efficient *in vivo* gene transfer, since there is no ideal vehicle system. Current delivery methods can be divided into viral and nonviral methods. While viral vectors are used for their higher cell transduction efficiency, nonviral methods remain attractive because they are less toxic, much easier and cheaper to produce, safer, and tissue-specific in some cases, and do not show DNA insert size limitations. Among the different nonviral strategies currently under study, *in vivo* DNA electrotransfer or electrotransfection (terms used synonymously in this chapter) has proven to be one of the most efficient and simple methods. Electrotransfection is based on cell membrane electroporation mediated by electric field delivery. This electroporation technology has been used in a more general way for drug delivery to targeted cells, including in the cases of the anticancer drug bleomycin or of other foreign molecules such as proteins, oligonucleotides, or RNAi.

This chapter gives a short description of the *in vivo* electroporation technique (mainly for DNA delivery), together with some reported applications in gene therapy, vaccination, or functional study.

11.1 Theory and Mechanisms

11.1.1 History

The *in vivo* electrotransfer technique is based on early studies of *in vitro* electroporation. Electroporation is a physical method that overcomes the barrier of the cell membrane for intracellular delivery of molecules. The cell membrane is a nonpermeable lipophilic barrier that controls exchanges of molecules between the cytoplasm and the external medium. A few hydrophobic molecules are able to enter the cytoplasm by crossing the lipidic bilayer, whilst others have to enter by specific transporter systems, but the majority of hydrophilic molecules are unable to enter the cell. In this context, the issue was to make macromolecules enter a variety of living cells.

In 1982, E. Neumann [1] first demonstrated that DNA could be introduced into living cells with the aid of electric pulses. By application of short and intense electric pulses, it was possible to create transient permeabilization of the cell membrane, facilitating the entry of any molecule. Since this result, this technique – called electropermeabilization or electroporation – has been routinely used *in vitro* on both prokaryotic and eukaryotic cells, and then on living animals. Electric parameters have been optimized in order to permeabilize cells transiently and to obtain good cell survival rates [2].

11.1.2 Mechanism of in vitro Electrotransfection at the Scale of a Single Cell

The technique of electroporation has been used for nucleic acid transfer since the 1980s, although the mechanism by which it occurs has not been completely elucidated. We focus here on studies of the mechanism underlying DNA electrotransfection at the level of the entire cell. This mechanism consists mostly of cell permeabilization and DNA uptake through electrophoresis.

11.1.2.1 Permeabilization

At the cellular level, the consequences of exposure to electric pulses have been widely studied. One can consider the cell as a conductive body (cytoplasm) surrounded by a dielectric layer (surface membrane). When an electric field is applied to the cell, the resulting current induces accumulation of electric charges at the cell membrane and modulates the cell transmembrane potential. The resting cell transmembrane potential ($\Delta\Psi_0$) is approximately –70 mV. If the transmembrane potential exceeds a critical threshold value, structural changes occur at the cell membrane (Figure 11.1).

The transmembrane potential induced by an applied electric field DY_i is described by Schwann's equation:

$$\Delta\Psi_i = F \cdot g(\lambda) \cdot r \cdot E \cdot \cos\theta \qquad (1)$$

where F is a shape cell factor, g(λ) a parameter depending on the membrane conductivity, r the cell radius, E the field intensity applied, and θ the angle between the field direction and the direction of the perpendicular to the membrane at the considered point of the cell. The membrane becomes permeable when the sum

$$\Delta\Psi_0 \text{ (resting transmembrane potential)}$$
$$+ \Delta\Psi_i \text{ (induced transmembrane potential)}$$

reaches a threshold value $\Delta\Psi_t$, which is around 200 mV for a cell [3]. The major characteristic of electropermeabilization is this threshold transmembrane potential $\Delta\Psi_t$. As the bilayer membrane is a common feature of cells, $\Delta\Psi_t$ is similar for various cell types. According to Schwann's equation, one can assume that the threshold intensity of the applied electric field necessary to obtain membrane permeabilization (E_t) is inversely proportional to the cell radius. Indeed, permeabilization is obtained at much lower electric fields for eukaryotic nucleated cells than for bacteria, which are smaller and require very high (~6000 V · cm^{-1}) electric fields for electrotransfection.

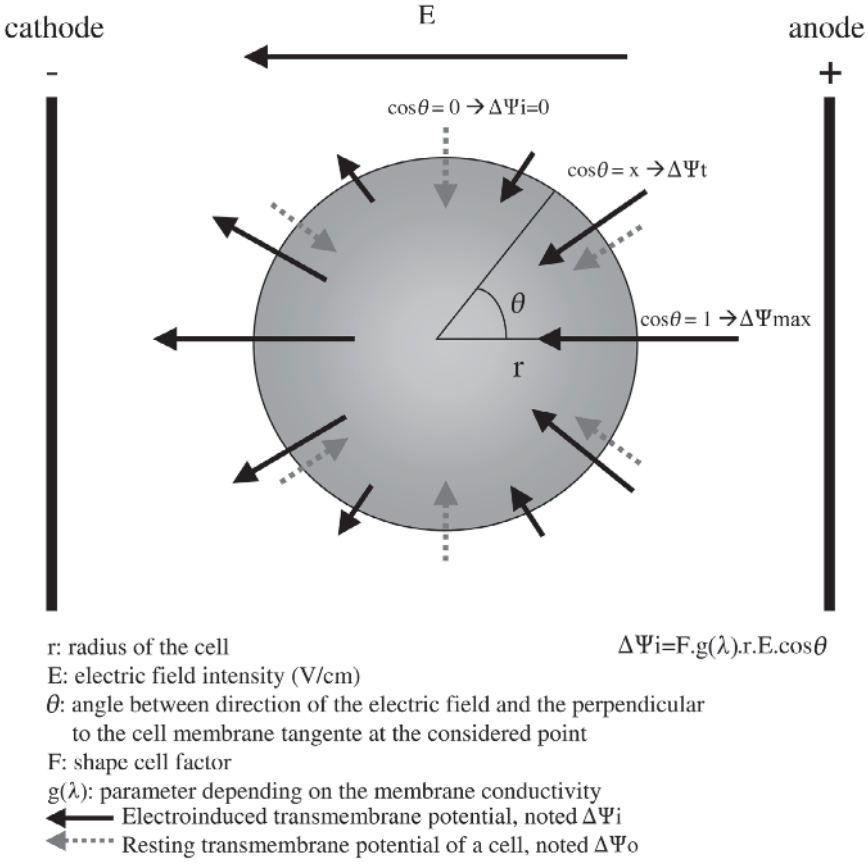


Figure 11.1 Effect of an external field applied on a living cell.

11.1.2.2 Uptake of DNA

The molecular mechanisms underlying DNA uptake during electropermeabilization are still largely unknown. Different models have been proposed in the case of mammalian cells [4].

One model suggests that electropermeabilization results in the formation of longlived "electropores" [1, 5]. Plasmid DNA would then perhaps cross the membrane after a binding step at the cell surface.

Another theory suggests that plasmid DNA crosses the membrane during the delivery of the electric pulses, due to electrophoretic forces associated with the applied field. Evidence supporting this electrophoretic effect has been offered by different groups: Klenchin et al. demonstrated the necessity of the presence of DNA within the cell culture medium during the electroporation procedure [6]. They also showed that transfection efficiency varied depending on whether the polarity of the applied electric field induced DNA electrophoresis towards the cells or away from the cells. Sukharev et al. demonstrated that short duration high voltage (HV) pulses promote membrane poration but not transfection efficiency, while long duration low voltage (LV) pulses promote the movement of DNA into the cell but not poration [7].

A fluorescence microscopy study at the single cell level [8] revealed that millisecond electric pulses induced interaction between the electropermeabilized membrane and DNA which is electrophoretically pushed against the cell surface: plasmid was seen to accumulate at the cell membrane but did not immediately move into the cytosol. DNA had to be present during electropulsation but crossed the electropulsed membrane only in the minute following it [9]. The same group recently demonstrated the relationship between the DNA/membrane surface interaction and gene transfer efficiency: increasing the surface for interaction of DNA with the membrane resulted in higher gene expression [4].

In summary, the exact mechanism of DNA electrotransfection into cells is not fully elucidated. It certainly involves both a cell permeabilization and an electrophoretic effect, but at the moment the timing of these events and the respective contribution of each in nucleic acid transfer are not known.

11.1.3 Mechanism of in vivo DNA Electrotransfer

While several studies of *in vitro* electroporation for the delivery of molecules to various living cell types (including eukaryotic cells) were reported during the 1980s, *in vivo* electroporation appeared only in the early 1990s. The first relevant *in vivo* application of electropermeabilization was demonstrated by the cellular uptake of the antibiotic and chemotherapeutic agent bleomycin into tumors [10]. Bleomycin is an antineoplastic agent that causes single-strand and double-strand breaks in DNA. Bleomycin's efficiency is dependent on the intracellular concentration, but this drug enters cells poorly. It was shown that better penetration of bleomycin was obtained by application of electric pulses to tumors, providing enhanced cytotoxicity. Since then, this technique [11, 12] has become well established under the name of

electrochemotherapy. Electrochemotherapy has also been applied with, for example, another anticancer drug, cisplatin, both in clinical trials on malignant melanoma skin metastases [13] and for veterinary use in horses [14].

Besides electrochemotherapy, the last few years have seen electric pulse-mediated gene transfer as a rapidly emerging and promising technique, under the name of electrotransfer. *In vivo* DNA electrotransfer, consisting of the injection of plasmid DNA into a targeted tissue and the application of a series of electric pulses, is a simple physical technique for gene delivery into various mammalian tissues.

As in the *in vitro* case, the exact mechanism of *in vivo* DNA electrotransfer is not fully elucidated. Most results have provided evidence that the mechanism of DNA electrotransfer *in vivo* can be regarded as the same mechanism as described previously, extended to a whole tissue: permeabilization is triggered when the local field reaches a critical value, and *in vivo* DNA electrotransfer is a multistep process. Here we describe a few of the reported *in vivo* mechanistic studies.

In 1999, Mir et al. ([15], Chapter 12) showed that electric pulses increased gene transfer not only by cell permeabilization but also through a direct active effect on the DNA molecule, promoting DNA migration and cellular uptake. They considered the uptake of a radioactive marker (^{51}Cr-EDTA) as evidence of muscle-fiber permeabilization on one hand, while on the other hand viewing DNA expression as evidence of DNA uptake. They showed that addition of ^{51}Cr-EDTA shortly after the delivery of electric pulses resulted in uptake, whereas gene transfer did not occur when DNA was added after pulse delivery.

Evidence of the association of cell permeabilization and DNA electrophoresis during electrotransfer has been observed *in vivo* in mouse skeletal muscle in study of combinations of LV nonpermeabilizing pulses of long duration (electrophoretic effect) and of HV pulses of short duration (permeabilizing pulses) [16]. Only a sequence of one HV pulse (800 V $\cdot$ cm^{-1}, 0.1 ms) followed by electrophoretic pulses (80 V $\cdot$ cm^{-1}, 80 ms) resulted in highly efficient gene transfer. Further study [17] demonstrated that the role of the HV pulses is limited to cell permeabilization, whilst the LV pulses have a direct effect on DNA. More precisely, a NMR imaging study showed that when muscle was subjected to a series of electrical pulses efficient for electrotransfer, the zone of permeabilization to the gadolinium complex Gd-DTPA (a NMR contrasting agent) was similar to the zone of expression of an electrotransfered plasmid coding for the β-*galactosidase* reporter gene [18].

Cell permeabilization and DNA electrophoresis may not be the only mechanisms contributing to DNA translocation into the cell. Rols et al. [19], for example, have suggested the importance of energetic metabolism (ADP and ATP) to allow crossing of DNA through the cell membrane and its migration towards the nucleus. Satkauskas et al. [20], in another study on intramuscular electrotransfer, proposed a DNA uptake mechanism based on receptor-mediated endocytosis, although this could not explain electrotransfer. It was also shown in this study that constant gene expression could be observed if the pulses were applied up to 4 hours after DNA injection, although other results showed that most of the injected DNA had been lost by that time [21]. We have recently proposed the hypothesis that, after intramuscular injection, DNA is rapidly partitioned between at least two compart-

ments: a major part of the DNA stays in a first compartment, where it is rapidly cleared and degraded, whereas a small part of DNA constitutes the electrotransferable pool of DNA and is more stable [22].

To conclude concerning the molecular mechanisms underlying *in vivo* DNA electrotransfer, it appears that this is a phenomenon still under investigation. It is most probably a multistep process including DNA injection and distribution, cell permeabilization, and DNA transfer facilitated by DNA electrophoresis. However, better understanding of the details and the contributions of each phase of this complex process should permit further designs of more effective electrotransfer strategies.

11.2
In vivo DNA Electrotransfer in Practice

For electrotransfer, a solution of plasmid DNA is injected into the targeted tissue and electric pulses are delivered by means of two electrodes (usually needles or plates) positioned on each side of the injection site and connected to a pulse generator (Figure 11.2). This technique is widely used in a large variety of tissues with enhanced gene expression, as compared to naked DNA injection.

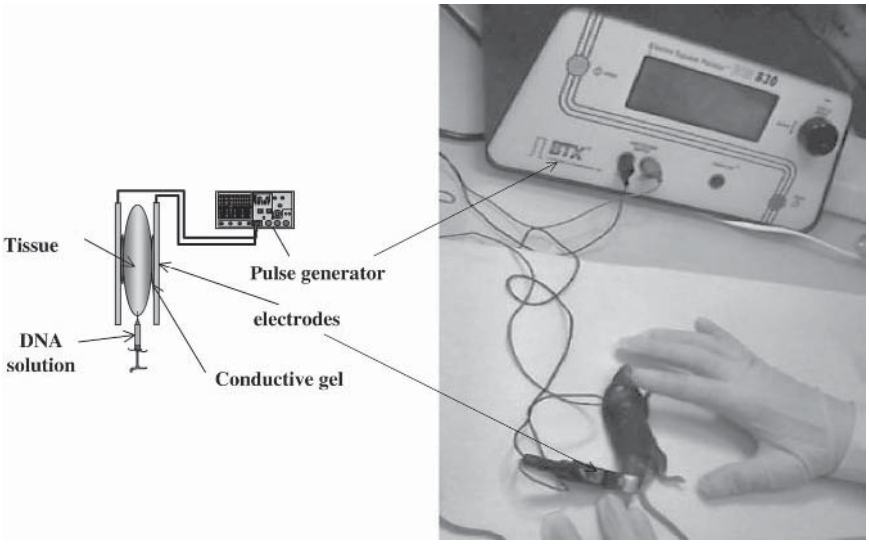


Figure 11.2 Achieving electrotransfer: local injection of plasmid DNA followed by appropriate electric pulse delivery around the injection site.

11.2.1 Device and Electrical Parameters

The choice of electrode design depends on the targeted tissue and on the size of the animal being electrotransfered. In any case, the type of electrode is of critical importance and need to be carefully considered. For skin DNA electrotransfer, for example, Zhang et al. [23] have compared two noninvasive techniques on mouse and human skin, using either meander (an array of interweaving electrode fingers of alternative polarity) or caliper electrodes. They observed that both electrodes were equally effective and more patient-friendly than needle electrodes. When DNA electrotransfer is applied to small animal tissues such as skeletal muscle, tumor, or liver, the majority of groups tend to use plate electrodes, consisting of end-plates attached to a caliper. Plate electrodes can indeed be easily applied externally to either side of the tissue of interest. For larger animals, needle electrodes are more commonly used, as plate electrodes may be unsuitable for electrotransfer because of the high electric fields that would be needed. In that case electrodes may also consist of needle arrays (six or more needles) to allow multiple injection and electroporation sites.

More precisely, knowledge of the electric field intensity and distribution for effective gene transfer *in vivo* is of great importance both to obtain cell permeabilization and to reduce cell toxicity. The electric field pattern varies with the tissue and the electrode type, resulting in a varying effective intensity of the field (in $V \cdot cm^{-1}$) in the treated area. It appears that the field intensity distribution is more homogeneous for plate than for needle electrodes and that the electric field obtained in the case of needle electrodes is lower than for plate electrodes (if identical voltages are applied). Needle electrodes allow the delivery of the electric field in a precise area and at a chosen depth [24]. For each different species and tissue, it will be necessary to determine the respective reversible (permeabilization) and irreversible (cell damage) threshold field intensity values to obtain optimal electric conditions for gene transfer with minimized toxic effects. In this context, Miklavcic et al. [25] developed a model combining numerical predictions and experimental observations to determine these threshold values in the case of needle electrodes for drug delivery on rat liver.

Different types of electric pulses can be delivered by commercial electropulsators. Exponential pulses are still often used for *in vitro* experiments. The exponential time constant depends on the set capacity and the resistance of the media. Square wave electric pulses are preferred for *in vivo* experiments, since the voltage and duration of the pulses can be set independently of the electrical resistance of the tissue. One advantage of DNA electrotransfer is the possibility to adapt the pulse protocols to different tissues. Various efficient protocols have been published by different groups using either low voltage (100–300 V) long duration (4–50 ms) pulses into melanoma [26] and muscle [27], for example, or high voltage (400–1200 $V \cdot cm^{-1}$) short duration (95–300 μs) pulses into liver [28], tumors [29], or muscle [30]. As the electrical parameters depend both on the delivery methodologies and on the electrical characteristics of targeted tissues, this allows a great variety of protocols (see Table 11.1 for some examples in muscle).

Table 11.1 Variety of protocols for electrical pulse delivery in muscle.

Species	*Electrodes*	*Conditions of electroporation*	*Gene*	*Reference*
Mouse	Plate	200 V/cm; 8 pulses; 20 ms; 1–2 Hz	Luc, hSeAP, hFIX	Mir 1999, Bettan 2000
	Plate	200 V/cm; 6 pulses; 20–50 ms; 1 Hz	Luc, LacZ	Miller 2004
	Plate	130 V/cm; 6 pulses; 60 ms; 10 Hz	Luc, LacZ	Bertrand 2003
	Wire	90 V/cm; 1000 biopolar pulses; 10 trains 1 s interval	EPO, HSeAP	Cappeletti 2003
Rat	Needle	200 V/cm; 8 pulses; 50 ms	EPO	Maruyama 2001
Rabbit	6-Needle	200 V/cm; 6 pulses; 50 ms; 1 Hz	Hepatitis B surface antigen	Widera 2000
Guinea pig	6-Needle	200 V/cm; 6 pulses; 50 ms; 1 Hz	Hepatitis B surface antigen	Widera 2000
Pig	Needle/plate	100–200 V/cm; 6 pulses; 60 ms; 1 Hz	SeAP, growth hormone releasing hormone (GHRH)	Draghia-akli 2003
	6-Needle	200 V; 6 bipolar pulses; 20 ms; 5 Hz	Bovine herpesvirus glycoprotein D (gD) and hepatitis B surface antigen	Babiuk 2002
Sheep	Separate double-needle electrodes	150–200 V/cm; 1000 pulses; 200 μs; 10 trains 1s interval	GFP, *H. Contortus* antigens	Scheerlinck 2004
Goat	Combined single-needle syringe	150–200 V/cm; 1000 pulses; 200 μs; 5–10 trains 2s interval	mycobacterial antigens (MPB70, Ag85B, Hsp65)	Tollefsen 2003
Cattle	Separate double-needle electrodes	150–200 V/cm; 1000 pulses; 200 μs; 5–10 trains 2s interval	mycobacterial antigens (MPB70, Ag85B, Hsp65)	Tollefsen 2003
Rhesus macaque	6-Needle	200 V/cm; 6 pulses; 50 ms; 1 Hz	HIV-1 Gag, Env	Otten 2004

11.2.2
DNA Electrotransfer and Toxicity

Optimal conditions for plasmid DNA electrotransfer into a tissue are the result of a compromise between efficient plasmid transfer and minimal cell toxicity. One of the disadvantages of electrotransfer is the potential damage associated with the procedure. This toxicity may involve different parameters: permeabilization is a main factor of toxicity, since external media diffuse into cells and modify their internal media composition. Internal medium may also leak out the cell. This is reduced when the duration and the level of permeabilization are minimal. Another toxic effect is oxidative stress due to the generation of free radicals induced near the membrane by electropermeabilization [2]. However, we have recently shown with stress/toxicology microarrays that the delivery of electric pulses for DNA electrotransfer to mouse muscle does not induce the expression of stress-related genes [31]. Furthermore, it was shown on a muscle model that electrotransfer induces plasmid-dependent muscle lesions containing necrotic myofibers, although electrotransfered muscles were indistinguishable from untreated controls at day 56 [32]. Another recent study confirmed this result, suggesting that gene electrotransfer associated muscle damage mainly arises from the intracellular presence and expression of plasmid DNA [33]. Finally, *in vivo* delivery of electric pulses to tissues can induce vascular effects [34]. Bertrand et al. [35] recently showed that electroporation induced only very transient phenotypic and morphological alterations of muscle fibers in transgenic mice harboring a transgene under the control of a fiber-specific and nerve-dependent promoter, although the process resulted in profound but transient alteration of muscle transcriptional status. In the case of the muscle fibers, seven to ten days after DNA electrotransfer seem to be necessary for a normal physiological state to be recovered.

11.2.3
Plasmid Biodistribution

The importance of access of plasmids to targeted cells has been demonstrated in different reports. It has been shown, mostly in skeletal muscle, that improved plasmid distribution results in an increase in DNA expression. Improved plasmid distribution was achievable by preinjection of a sucrose solution, which created spaces between muscle fibers [36], or by pre-treatment with hyaluronidase [37], which breaks down components of the extracellular matrix containing hyaluronan and collagen [38]. Moreover, this improved gene transfer with hyaluronidase pretreatment allowed the use of lower voltages, resulting in a reduction in muscle damage [39]. Molnar et al [40], for example, showed (by β-Gal histochemistry) an increased muscular transfection efficiency of 150–370% after pretreatment with hyaluronidase and electrotransfer (175 $V \cdot cm^{-1}$) in different strains of mice. Poly-L-glutamate, an anionic polymer that may increase intracellular uptake and trafficking of plasmid and/or reduce degradation, has also been used in mouse muscle to enhance transgene expression [41].

Table 11.2 Variety of tissues targeted for electrotransfer.

Tissue	*Species*	*Gene*	*Reference*
Muscle			see Table 1
Skin	Rat	EPO	Maruyama 2001
	Mouse	Luc, LacZ	Zhang 2002
Tumor	Rat	LacZ	Nishi 1996
	Mouse	LacZ	Rols 1998
	Mouse	Luc	Wells 2000
	Mouse	Luc, LacZ	Bettan 2000
	Rat	Luc	Heller 2000
	Mouse	MBD2/demethylase	Ivanov 2003
	Rat	GFP	Cemazar 2004
Liver	Rat	Luc	Heller 1996
	Rat	GFP	Suzuki 1998
	Mouse	Luc, LacZ	Liu 2002
Lung	Mouse	Luc, LacZ	Dean 2003
Cartilage	Rat	Luc	Ohashi 2002
	Rat	GFP	Grossin 2003
Embryos	Mouse, chicken	LacZ	Itasaki 1999
	Chicken	GFP	Luo 2004
Kidney	Rat	LacZ, Luc	Tsujie 2001
Brain	Mouse, chicken	GFP	Inoue 2001
	Mouse	GFP	Saito 2001
	Xenopus tadpole	GFP	Haas 2002
	Honeybee	GFP	Kunieda 2004
Carotid artery	Rabbit	Luc	Matsumoto 2001
Testis	Mouse	CAT, Luc, LacZ	Muramatsu 1997
Ovary	Mouse	LacZ	Sato 2003
Cornea	Mouse	IL-6	Blair-Parks 2002
	Rat	GFP	Oshima 2002
Retina	Rat	GFP	Dezawa 2002
	Mouse, rat	GFP	Matsuda 2004
Conjunctiva	Rabbit	MMP-3	Mamiya 2004
Spinal cord	Rat	GFP	Lin 2002
Spleen	Mouse	Luc, GFP, hSeAP, IFNγ	Tupin 2003
Bladder	Rat	Luc, GFP, Muscarinic receptor	Otani 2004

The therapeutic efficiency of a drug is related to its blood concentration and halflife. Particularly for secreted proteins, it is usual to predict their efficiency from their plasmatic kinetics. One of the major advantages of gene transfer compared with the administration of recombinant proteins is the capacity quickly to reach a steady-state level of circulating protein secreted by the transfected organ. In contrast, the administration of recombinant proteins first results in a peak of concentration, which may be within the toxicity zone, and this then rapidly falls to subtherapeutic levels. Different kinetics of gene expression have been described after DNA *in vivo* electrotransfer in skeletal muscle.

Long term kinetics of expression have been observed for different transgenes, such as human secreted alkaline phosphatase (hSeAP), the luciferase reporter gene [15], human factor IX (hFIX) in SCID mice [42], or murine erythropoietin (mEpo) in immunocompetent mice [43]. By following luciferase expression with a CCCD (conductively connect charge-coupled device) camera, an imaging technique that allows *in vivo* kinetic study without sacrificing the animals, it was observed that gene expression increased with time during the first few days, and then stayed at comparable levels for at least 70 days [44]. Expression has been shown to last for up to a year [42], raising hopes in the gene therapy field.

Other genes, in contrast, have shown very short profiles of expression. As an example, murine IL-10 cytokine could not be detected in the blood 15 days after mouse muscle electrotransfer, although some expression was still remaining in the muscle [45]. Immunoinflammatory effects might explain rapid cytokine decline.

In some cases transgene expression also showed a gradual decline in mice [40], although the reasons are not well understood. It has been suggested that promoter attenuation might occur [46]. On the other hand, Cappeletti et al. [21] showed that the amount of plasmid DNA in the muscle decreases with roughly the same profile as the circulating protein.

11.3
Targeted Tissues

In vivo electrotransfer appears to be a simple and efficient gene transfer method and has been applied in recent years to a variety of tissues, including skeletal and cardiac muscles, skin, liver, lung, kidney, joints, spinal cord, brain, retina, cornea, vasculature, and others (Table 11.2). Different electroporation conditions are applied depending on the targeted tissue, since gene transfer is highly dependent on tissue organization and transfected cell size.

11.3.1
Skeletal Muscle

Electrotransfer in skeletal muscle was discovered independently by three teams [15, 27, 47, 48]. Skeletal muscle is the most widely targeted tissue for electroporation because it offers several advantages: (1) it constitutes a large easily accessible volume

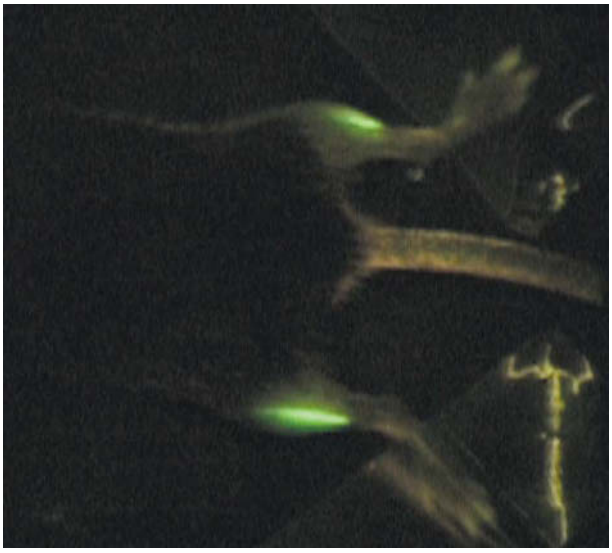


Figure 11.3 Green fluorescent protein (GFP) expression in skeletal mouse muscle three weeks after intramuscular injection and electrotransfer of 40 μg of plasmid DNA encoding for GFP gene (200 V · cm^{-1}, 8 pulses, 20 ms duration).

(Figure 11.3), (2) muscle fibers have long lifespans, as they are postmitotic cells, which potentially allows long term expression in transfected cells in the absence of regeneration due to injury or cytotoxic immune response [15], and (3) skeletal muscle is made up of thousands of cylindrical muscle fibers bound together by connective tissue through which run blood vessels and nerves, constituting an abundant blood vascular supply [49]. Skeletal muscle is therefore able to produce secreted proteins that can easily reach the blood circulation (for a review see [50]).

For all these reasons, muscle is a target of choice for gene therapy. Indeed, the persistence of DNA in an episomal state for months and the ability of skeletal muscle to secrete proteins allow multiple therapeutic approaches such as direct gene transfer for muscle disorders, DNA vaccination, or systemic delivery of therapeutic proteins.

11.3.2 Tumor Tissue

In vivo electropermeabilization, with increases both in drug delivery and in gene transfer, has been extensively reported in malignant tumors. In electrochemotherapy, tumor cell permeabilization drastically increases the uptake of antitumor drugs such as bleomycin [51, 52] or cisplatin [53], resulting in great improvements in the efficacies of these drugs. The electric conditions involved short (100 μs) and intense (800 to 1500 V · cm^{-1}) electric pulses. Concomitantly, improvement in gene transfer has been demonstrated with reporter gene in rat brain tumors by combining

in vivo electroporation and intraarterial plasmid DNA injection [29], and by intratumoral injection in mouse melanoma tumor with electric pulses of 800 V · cm^{-1} and 5 ms duration [26], in mouse mammary tumors with electric pulses of 400–2300 V · cm^{-1} and 1 ms duration [54], in rat liver tumors with electric pulses of 1000–1500 V · cm^{-1} and 100 μs duration [55], and in various other type of human and murine tumors (lung and colon carcinoma, melanoma and sarcoma) with electric pulses of 400–600 V · cm^{-1} and 20 ms duration [56].

11.3.3 Skin

The skin is an attractive target tissue for gene transfer for several reasons: (1) it is an accessible tissue, which facilitates *in vivo* gene delivery, (2) keratinocytes function as synthetic and secretory cells allowing systemic delivery [57], (3) skin contains potent antigen-presenting cells (APCs) useful for DNA vaccination, and (4) epidermal cells have short lifespans, which can be useful for treatments requiring relatively short periods of gene expression, such as DNA vaccination.

Divided into two main layers, epidermis and dermis, however, the skin appears to be a difficult target tissue for efficient gene transfer. Indeed the skin's outer layer, the stratum corneum, consists mainly of keratin and so acts as the first and most important barrier of the skin [58]. Several methods of gene transfer have been reported (for a review see [59]), including intradermal naked DNA injection, "gene gun", or skin patch. Among them, *in vivo* electrotransfer appears to be an effective technique to enhance transdermal gene delivery [60]. Dujardin et al. [61] demonstrated that the effects induced by square or exponential wave pulses on the skin were relatively mild and reversible, with no inflammation or necrosis, and permitted transient impairment of the barrier function of the skin. Zhang et al. [23] studied noninvasive *in vivo* DNA electrotransfer using either meander or caliper electrodes on mouse and human skin. With DNA electrotransfer they observed cutaneous luciferase and β-galactosidase transgene expression several hundred times higher than could be obtained simply by intradermal injection alone.

11.3.4 Liver

The liver is a very attractive target organ for gene therapy as it is physically a large target and is also the site of production for many enzymes [62].

Electrotransfer in rat liver was efficiently achieved by Heller et al. [28], with needle-array electrodes delivering six pulses of 99 μs in duration and of 1000 V · cm^{-1}, and by Suzuki et al. [63], with tweezer-type electrodes delivering eight pulses of 50 ms in duration at 50 V, pressed directly on the surface of a liver lobe in situ.

Interestingly, Liu et al. [64] have shown that the combination of systemic injection of DNA via tail vein and electrotransfer of the liver produces efficient gene delivery to hepatocytes. The systemic injection via tail vein, called hydrodynamics transfection (see also Chapter 9) consists of fast injection of a large volume (0.1 mL per g

body weight) of a plasmid solution into the tail vein [65]. In this study the systemic administration was compared with local injection, and it appears that gene expression was enhanced with systemic injection as compared to local injection, resulting in a broader distribution of gene expression. This combined technology needs to be improved further [66] but might have interesting applications even in other tissues.

11.3.5 Lung

One characteristic of lung that makes it amenable to gene transfer is that it can be targeted both from the vascular surface and from the airway epithelial surface. With current delivery systems – either systemic administration of liposome–DNA complexes by intravenous injection or delivery of complex and viruses via the airways – gene delivery to all lung cell types remains very difficult to achieve, due to numerous physical and biological barriers. Recently, Dean et al. showed that delivery of naked DNA to mouse lung by electrotransfer resulted in high levels of gene expression over one week with no trauma or damage, either macro- or microscopically [67]. DNA was delivered to the lung by intratracheal injection, and an electric field was then applied across the chest of the animal with eight square wave pulses of 10 ms duration at a field strength of 200 $V \cdot cm^{-1}$. Gene expression was seen predominantly in the peripheral alveoli and in both the epithelium and deeper cells. Electrotransfer could thus become an attractive technique to bypass the barriers to other gene delivery techniques described above and to provide gene transfer and expression to all the cells in the lung, including airway and alveolar epithelial cells, airway smooth muscle cells, and vascular endothelial cells.

11.3.6 Vasculature

An efficient and safe nonviral gene transfer technique for the treatment of cardiovascular disease in humans would be of great interest. In this context, Matsumoto et al. [68] demonstrated that electrotransfection was applicable for arterial *in vivo* gene transfer, under optimized electrical conditions (plate electrodes with a 1 mm distance between them, 10 pulses of 200 $V \cdot cm^{-1}$ and 20 ms duration). These conditions resulted in efficient gene transfer in rabbit carotid artery for at least 14 days, although voltage-dependent damage in the arterial wall was apparent, mostly at 300 $V \cdot cm^{-1}$.

11.3.7 Eye

Eye tissues are a recently developed target for *in vivo* DNA electrotransfer. Indeed, this technique appears to be not only a promising strategy for gene therapies in the eye, but also a powerful means of elucidating the mechanisms of eyes diseases. Electric pulse-mediated gene transfer to corneal stromal cells, for example, has

recently been achieved with good results with a GFP reporter gene [69]. Optimal electrotransfer conditions were eight pulses of 20 V · cm^{-1} for 20 ms duration, with no inflammation observed. In addition, luciferase and green fluorescent protein genes have been delivered to multiple cell layers within the mouse cornea with extremely high levels of gene expression and almost no inflammatory response or tissue damage when electroporation conditions were eight pulses of 200 V · cm^{-1} and 10 ms duration [70]. Recently, Matsuda et al. [71] have used *in vivo* DNA electrotransfer in rodent retina for loss- and gain-of-function studies, with use of a GFP reporter gene and the RNAi technology. This technique has also been applied to rabbit conjunctiva with the matrix metalloproteinase-3 gene (MMP-3) [72].

11.3.8 Embryos

In vivo DNA electrotransfer in embryos is a fascinating new approach by which gene expression, regulation, and function in developmental systems can be studied (for a review see [73]). For embryos, low fixed voltages are used (25–65 V · cm^{-1} for chicken embryos, 65–225 V · cm^{-1} for mouse embryos [74]) to prevent destruction of tissue architecture and to permit cell viability. A comparison of three nonviral transfection methods (microparticle bombardment, lipofection, and electroporation) suggested electroporation as the method giving the strongest expression of a LacZ reporter gene [75].

11.3.9 Cartilage

In vivo DNA electrotransfer to intraarticular tissues has been developed in order to improve gene therapy of joint diseases such as rheumatoid arthritis, osteoarthritis, and cartilage injuries. Ohashi et al. [76] reported efficient gene transfer into synovium of rat knees after intraarticular injection of a luciferase reporter gene followed by electric pulse delivery with an optimal electric field of 215 V · cm^{-1} (150 V/0.7 cm), comparable to that reported by Mir et al. in skeletal muscle [15]. More recently, Grossin et al. [77] observed high level and stable long term GFP expression in the rat patellar cartilage after *in vivo* DNA electrotransfer at an optimal electric field of 250 V · cm^{-1}.

11.3.10 Gonads

DNA electrotransfer into testis has been tested: after a surgical procedure to expose testicular tissues, DNA was injected into testis and square electric pulses were applied (8 pulses, 25 V/50 ms or 50 V/10 ms, unfortunately no indication of distance between electrodes). CAT and LacZ reporter gene were expressed transiently in a dose-dependent fashion in seminiferous tubules in some spermatogenic-like cells deep inside the testis. This technology might ultimately provide a novel approach

for the production of transgenic animals [78]. More recently, this technique has been also used with chicken testis, showing strong but transient expression in the testis [79].

Intraovarian injection of plasmid DNA and subsequent electrotransfer has been tested and has proven to be an efficient technique to deliver genes to the ovarian cells, including follicular cells and oocytes of mice. The ovary was held between a tweezers-type electrode and square electric pulses were applied eight times at 50 V with a constant time of 50 ms. These treatments produced no noticeable damage to the ovary at the histological level, and the technique might be useful for testing the function of genes of interest during oogenesis [80].

11.4 Therapeutic Applications

11.4.1 Intramuscular Electrotransfer

11.4.1.1 Ectopic Secretion of Proteins

Several studies with reporter genes have highlighted the potential of DNA electrotransfer in skeletal muscle to produce sustained high levels of circulating protein in the blood. Skeletal muscle is a good candidate as an neoendocrine tissue for expression of cytokines, growth factors, or clotting factors, for example, as therapeutic levels of secreted proteins can be reached [42, 43]. Two groups have recently shown that EPO secretion after muscle electrotransfer of a plasmid encoding for the EPO gene assisted by the CMV promoter or the tetracycline-inducible promoter Tet-on results in improved erythropoiesis, increase in red cells halflives, and high hematocrit for several months in a β-thalassemic mouse model [81, 82]. This therapeutic effect is based on the induction of β-minor hemoglobin gene with bone marrow stimulation at very high EPO concentrations and could be developed in humans as a treatment for β-thalassemia by induction of the human fetal γ-hemoglobin subunit. Anemia linked to renal failure is also a potential target for EPO gene therapy, as shown by Maruyama et al. [83]. Skeletal muscle has also been used for production of cytokines, resulting in improved survival in a mouse viral myocarditis model [84] or in rat induced myocarditis [85]. The antiinflammatory cytokine IL-10 showed interesting properties in an atherosclerosis model [86].

A very interesting disease model illustrating the potential of plasmid intramuscular electrotransfer is collagen induced arthritis (CIA), a mouse model of rheumatoid arthritis. It has been shown that IL-10 or IL-4 exhibited a protective role against CIA after intramuscular electrotransfer of the corresponding plasmid-borne gene assisted by the constitutive CMV promoter [87–89]. Two independent parallel studies showed that inhibition of the proinflammatory cytokine TNFα by muscle electrotransfer of plasmids encoding TNFα soluble receptors resulted in decreases in both the histological and the clinical signs of the disease [90, 91]. The benefits of this single treatment were comparable to those obtained with repeated

injections of the clinically used recombinant protein Ethanercept [91]. Recently, another group has demonstrated that inhibition of the action of the proinflammatory cytokine IL-1 by electrotransfer of a IL1 receptor antagonist encoding plasmid (a ligand of IL-1 receptor that does not induce any intracellular response) also resulted in significant improvements in arthritis [92].

HGF muscle secretion recently showed cytoprotective activity in a mouse acute liver injury model [93]. Another promising result was recently obtained by Prud'homme et al. [94], who showed protection against autoimmune diabetes by muscle secretion of a ligand of CTLA-4 (cytotoxic T lymphocyte antigen 4), a negative regulator of T cell activity.

Finally, another group has reported that factor VIII gene delivery by muscle electrotransfer produced a phenotypic correction of murine hemophilia A [95].

All these reports suggest that muscle DNA electrotransfer allows the production of therapeutic levels of secreted proteins and may merit further development on grounds of its simplicity, low cost, and safety.

11.4.1.2 **Muscle Disease Therapy**

It is encouraging to see that gene transfer by electroporation is also possible in fragile muscles such as dystrophic muscles. Indeed, expression of laminin $\alpha 2$ chain in dystrophic mouse muscle was obtained without extended muscle damage [96], although a loss of expression was observed with time, due to degeneration and regeneration of muscle. Moreover, it has been reported that highly efficient gene transfer of full length murine dystrophin could be obtained in mdx mouse (dystrophic mouse), and that the muscle damage induced by electrotransfer was not enhanced by the dystrophic phenotype [97, 98].

11.4.2 **Vaccination**

The principle of immunization is to induce both the generation of memory T and B cells and the presence of neutralizing antibody in the serum by injection of a foreign protein. In current vaccines, these foreign proteins are mainly live-attenuated pathogens (bacteria, viruses) or recombinant proteins. In both cases antigen preparation requires multiple purification and/or neutralization steps, before injection into animals or humans. The production of live-attenuated pathogens entails many safety considerations, and the isolation of enough pure antigen protein can represent a time-consuming and expensive procedure, sometimes also unfeasible. In this context, the observation that direct *in vivo* gene transfer of recombinant DNA resulted in expression of protein *in situ* gave rise to the development of DNA vaccines. Introduction of the gene encoding a protein directly into the skin [99] or muscle [100] of an animal elicits an immune response. In fact, this plasmid-based vaccine injection is an attractive approach as it provides several advantages over current vaccines. Plasmid DNA can be manufactured (Chapter 3) very cost-effectively (ultrapure DNA preparation on large scales is much easier than that of proteins and is the same whatever the plasmid), can be stored with relative ease

(no need for a "cold chain" to maintain the efficacy of the vaccine), and there are none of the safety concerns associated with live-attenuated vaccines or pathogens. The organism will itself produce the antigen inducing the immune response: the host acts as the bioreactor [101]. With "naked" DNA immunization it has been possible to obtain high titers of neutralizing antibodies in animals, but because of low or poorly reproducible gene transfer efficiency [102], multiple immunizations of high DNA doses are often required to achieve modest responses, particularly in primates [103]. One reason for the lack of efficacy of DNA vaccine in large animals and in the first human clinical trials seems to be inefficient uptake of DNA by cells in muscular tissue, which differs between small and large animal species.

In this context, electrotransfer greatly increases the potential of DNA vaccines, since it increases antigen expression levels by several orders of magnitude, and it has been demonstrated that the level of antibodies produced is related to the antigen expression level [104, 105]. While increased transfection efficiency and concomitant increased antigen expression may explain the increased immune response in animals treated with DNA injection and electrotransfer [103], damage to muscle cells and release of "danger signals" after electroporation may also contribute [106]. Different recent works have demonstrated the efficiency of electrotransfer in DNA immunization: antibody titers were increased in mice, rabbits, or guinea pigs after electrotransfer of a plasmid encoding a surface antigen of HBV virus [103], and this was also shown to be true in mice after electrotransfer of a plasmid encoding a tuberculosis protein [107]. We were able to show that electrotransfer of a plasmid encoding a hemagglutinin surface glycoprotein of the influenza virus induced a better immune response in mice than naked DNA injection [108]. For any human clinical application, but also for veterinary concerns, it was crucial to demonstrate that DNA injection and electrotransfer would also induce immune responses in larger animals. This technique has been applied to pig [109], sheep [106], or cattle [110], and improved immune responses were observed in all cases. The potency of an HIV DNA vaccine was enhanced in rhesus macaques by *in vivo* DNA electrotransfer [111].

Electrotransfer-mediated DNA vaccination can therefore be used to elicit immune response against foreign proteins, but this technique can also be used to produce monoclonal antibodies in muscle directly [112]. Injection of immunoglobulin genes as naked plasmid DNA into mouse skeletal muscle in combination with electrotransfer of the injection site yields correctly assembled serum monoclonal antibodies with intact specificity and effective biological functions [113, 114].

11.4.3 Cancer Gene Therapy

As cancer is a disease linked to somatic gene mutation, gene therapy seems to be an exciting area of research. However, effective and safe gene delivery methods for cancer cells are still lacking. Viruses are the most effective as far as transfection is concerned, but they may elicit immune responses and also raise some safety concerns. Nonviral vectors suffer from a lack of transfection efficiency. Out of 656

clinical trials in the field of cancer gene therapy to date, only 11 reached phase III, with only one using a nonviral vector (http://www.wiley.co.uk/wileychi/genmed/clinical/). If an efficient nonviral gene transfer method were to be developed, it would certainly allow great hopes for cancer gene therapy. Gene electrotransfer in accessible solid tumors is easy and rapid to perform, and we have shown it to be efficient [56]. Although the transfection efficiency is low in relation to that of viral vectors, it is a safe technique that can be repeated as much as necessary, resulting in growth of the number of transfected cells.

Strategies for cancer gene therapy can be divided into four basic concepts: (1) strengthening of the immune response against a tumor, (2) suicide gene strategies, (3) repair of cell cycle defects caused by loss of tumor suppressor genes or inappropriate oncogene activation, and (4) inhibition of tumor angiogenesis. Some of these strategies have recently been applied by *in vivo* DNA electrotransfer with encouraging results, showing the feasibility of this approach.

11.4.3.1 **Strengthening Antitumor Response**

Cytokine gene electrotransfer into tumors has been investigated intensively: IFN-α, IL-12, IL-18, or combinations of these genes have recently been shown to reduce tumor growth and to increase survival times in different tumor models [115–119]. In the case of IL-12, tumor eradication was observed in 40% of mice, which survived for a year. It has been suggested that IL-12 induces increases in IFN-γ, Mig, and IP-10, which trigger both the immune response and an antiangiogenic response [115]. Human IL-2 or murine GM-CSF electrotransfer into a model of human esophageal tumors grafted into nude mice suppressed the growth of these tumors and prolonged survival [120].

11.4.3.2 **Suicide Genes**

Suicide gene therapy is a promising strategy for cancer gene therapy. Suicide genes encode enzymes that convert nontoxic prodrugs into toxic metabolites that are lethal to cells. The herpes simplex thymidine kinase (HSVtk) gene is classically described as a model of the tumor suicide gene. Transfer of the HSVtk gene renders target cells sensitive to ganciclovir (GCV), an agent used clinically against cytomegalovirus and other viral infection. Once the HSVtk gene is transfected into tumors, cells produce the viral enzyme, which is capable of converting systemically introduced GCV into a phosphorylated product giving rise to a terminator of DNA synthesis (GCV-P-P-P). A combination of this HSVtk/ganciclovir technology with *in vivo* electrotransfer has proved to suppress the growth and metastasis of subcutaneously grafted mammary tumors in mice, although no complete regression was noted [121, 122]. To improve the antitumor effects of the method, Goto et al. recently tested repeated *in vivo* electrotransfer of a combination of HSVtk and IL-12 genes. Complete regression of tumors was frequently obtained with this combined therapy [123].

11.4.3.3 **Apoptosis-Inducing Genes**

Significant inhibition of tumor growth has also been obtained by intratumoral electrotransfer of TRAIL/Apo2 ligand, an apoptosis inducer [124], and by skeletal

muscle electrotransfer of a metalloproteinase-4 inhibitor [125]. Another encouraging result was obtained by electrotransfer into the liver of a liposome-encapsulated plasmid encoding the pro-apoptotic gene *bcl-xs* (member of the *bcl-2* family), with inhibition of the occurrence and growth of a rat hepatocellular carcinoma induced by *N*-nitrosomorpholine [126].

11.4.3.4 **Inhibition of Tumor Angiogenesis**

It is now well established that tumor growth and spreading are angiogenesis-dependent processes, so inhibition of angiogenesis is likely to be an effective anticancer approach. A antiangiogenic gene therapy approach has several advantages, including the potential for sustained expression and blood secretion of antiangiogenic proteins. As an example, intramuscular electrotransfer of fibstatin-encoding cDNA (a secreted antiangiogenic fragment containing the type III domains 12–14 of fibronectin) inhibits B16F10 tumor growth [127].

11.4.3.5 **Other Strategies**

It is known that methylation is an important mechanism for regulation of gene expression [128] and that cancer cells present aberrant methylation patterns. We have shown that intratumoral electrotransfer of an antisense of MBD2, an enzyme involved in DNA methylation, results in an important inhibition of tumor growth in a human tumor model grafted in nude mice [129]. Moreover, the combination of MBD2-antisense electrotransfer gene therapy with bleomycin electrochemotherapy has an additive inhibitory effect on the rate of tumor growth and a synergistic effect on the number of tumor-free animals relative to either monotherapy [130].

All these promising results show the potential of *in vivo* electrotransfer for cancer gene therapy, which could be used for surgically inaccessible tumors such as head and neck tumors. As the number of transfected cells is probably not sufficient, it is unlikely that tumor electrotransfer by itself will provide a cancer cure and, furthermore, the efficiency of gene transfer depends on the tumor tissue [56, 131]. Electrotransfer should, however, find application in combination with other strategies such as chemotherapy. As chemotherapy and gene therapy follow different mechanisms to kill cancer cells, synergy between them, in addition to different toxicity profiles, can reasonably be expected.

11.4.4
Electrotransfer as a Tool

In addition to its potential use in gene therapy, we think that DNA electrotransfer is a powerful laboratory complementary tool for study of *in vivo* gene expression in any given tissue.

Each tissue requires specific electrotransfer parameters that have to be empirically studied. This provides a tool for study of gene expression and function, in a spatially and temporally restricted manner, as is illustrated by the use of this technique in developmental biology [132]. In an excellent study, Saito and Nakatsuji performed embryonic mouse brain electrotransfer both *in utero* and *ex utero* [133] and showed

GFP expression in different targeted regions of the brain and visualized neuronal morphologies. It was also possible to cotransfect three different plasmids in the same cells. Electrotransfer was also performed on zebrafish for gene invalidation by a dominant-negative in a fin regeneration study [134]. A micropipette electroporation technique has also been used to transfect individual cells into the brains of intact Xenopus tadpoles [135]. *In vitro* and *in vivo* electrotransfer tools have also been used to decipher the transcriptional regulation of human skeletal muscle myosin heavy chain in muscle development and differentiation [136].

In vivo electrotransfer has proven to be a valuable tool for the study of gene regulation systems, such as the tetracycline system, which requires cotransfection of at least two plasmids in the same cell. Lamartina et al. [137], for instance, have studied the activity of novel doxycycline transactivators in a gene switch system, while we have studied a system based on hypoxia-responsive element and tetracycline transactivators [138]. We also studied a gene expression regulation system based on three plasmids with a combination of an antisense strategy and the tetracycline system [139].

It has also recently been demonstrated that dsRNA can be introduced by *in vivo* electrotransfer in tissue such as chicken embryos [140, 141], developing rat cerebellum [142], mouse muscle [143], or rodent retina [71], producing efficient RNA interference. The two approaches, DNA and dsRNA *in vivo* electrotransfer, provide powerful complementary methods for functional genomic analyses. Indeed, two strategies – gain- and loss-of-function analyses – are commonly used to study gene function *in vivo*. *In vivo* DNA electrotransfer appears to be a powerful tool for both strategies: gain of function might be obtained by overexpressing a gene of interest, while loss of function might be obtained by genetic antisense or RNAi knockdown. In this context, the development of *in ovo* electrotransfer in chicks has provided a simple and effective means of introducing nucleic acid molecules into chick embryos, which are classical model systems for developmental studies [144].

11.5 Conclusion

In vivo electrotransfer is a non-viral technique for reasonably efficient gene transfer. It offers the main following advantages:

- *Ease of accomplishment:* It is an easy and rapid technique, using locally injected plasmid DNA followed within a few minutes by appropriate delivery of electric pulses around the injection site. Animals are under anesthesia (total for small animals, local for large animals) during the whole procedure. External plate electrodes or invading needle electrodes may be used to deliver the electric pulses.
- *Safety in DNA production:* The DNA injected is plasmid DNA. It offers several advantages such as reduced toxicity associated with the reduction of appreciable deleterious immunological reaction by the host to the plasmid (although plasmid DNA has some immunostimulatory properties), together with easy, safe, and

cheap production. Moreover, multiple plasmids with large insert capacities can be successfully injected and electrotransfered.

- *Pleiotropic:* Any type of cell and tissue could theoretically be a target. Electrotransfer mediates DNA transfer to multiple cell types and cell layers within a tissue, no matter whether the cells are dividing or quiescent.
- *Tissue-specific:* The technique is tissue-specific, since the treatment is specifically located in the area exposed to the electric field and injected with the plasmid.
- *Efficient and reproducible:* Electrotransfer has proven to be one of the most efficient nonviral strategies, increasing gene expression by several orders of magnitude in various tissues and species and decreasing interindividual variability, thus allowing the modulation of transgene expression by variation of the amount of injected plasmid DNA.

Electrotransfer has been shown to be repeatable several times with no detrimental immune reaction. Its exact mechanism has yet to be elucidated, and improvements in its understanding can be expected from further studies. Parameters such as DNA biodistribution also have to be investigated further in order to optimize this technique. Still, electrotransfer appears to be a very promising technique, both in the field of gene therapy, and as a laboratory tool for functional genomics. Although no gene therapy clinical trial for electrotransfer is currently in progress, it is to be expected that it should soon happen. Some applications using skeletal muscle as an endocrine tissue to secrete proteins at therapeutic concentrations could be considered.

References

1 Neumann, E., et al., Gene transfer into mouse lyoma cells by electroporation in high electric fields. *Embo J.* **1982**, *1(7)*, 841–845.
2 Bonnafous, P., et al., The generation of reactive-oxygen species associated with long-lasting pulse-induced electropermeabilisation of mammalian cells is based on a non-destructive alteration of the plasma membrane. *Biochim. Biophys. Acta* **1999**, *1461(1)*, 123–134.
3 Teissie, J., Rols, M. P., An experimental evaluation of the critical potential difference inducing cell membrane electropermeabilization. *Biophys. J.* **1993**, *65(1)*, 409–413.
4 Faurie, C., et al., Effect of electric field vectoriality on electrically mediated gene delivery in mammalian cells. *Biochim. Biophys. Acta* **2004**, *1665(1–2)*, 92–100.
5 de Gennes, P. G., Passive entry of a DNA molecule into a small pore. *Proc. Natl. Acad. Sci. USA* **1999**, *96(13)*, 7262–7264.
6 Klenchin, V. A., et al., Electrically induced DNA uptake by cells is a fast process involving DNA electrophoresis. *Biophys. J.* **1991**, *60(4)*, 804–811.
7 Sukharev, S. I., et al., Electroporation and electrophoretic DNA transfer into cells. The effect of DNA interaction with electropores. *Biophys. J.* **1992**, *63(5)*, 1320–1327.

8 Golzio, M., Teissie J., Rols, M. P., Direct visualization at the single-cell level of electrically mediated gene delivery. *Proc. Natl. Acad. Sci. USA* **2002**, *99(3)*, 1292–1297.

9 Golzio, M., Rols, M. P., Teissie, J., *In vitro* and *in vivo* electric field-mediated permeabilization, gene transfer, and expression. *Methods* **2004**, *33(2)*, 126–135.

10 Belehradek, J., Jr., et al., Electropermeabilization of cells in tissues assessed by the qualitative and quantitative electroloading of bleomycin. *Biochim. Biophys. Acta* **1994**, *1190(1)*, 155–163.

11 Gothelf, A., Mir, L. M., Gehl, J., Electrochemotherapy: results of cancer treatment using enhanced delivery of bleomycin by electroporation. *Cancer Treat. Rev.* **2003**, *29(5)*, 371–387.

12 Mir, L. M., Orlowski, S., Mechanisms of electrochemotherapy. *Adv. Drug Deliv. Rev.* **1999**, *35(1)*, 107–118.

13 Sersa, G., et al., Electrochemotherapy with cisplatin: the systemic anti-tumour effectiveness of cisplatin can be potentiated locally by the application of electric pulses in the treatment of malignant melanoma skin metastases. *Melanoma Res.* **2000**, *10(4)*, 381–385.

14 Rols, M. P., Tamzali, Y., Teissie, J., Electrochemotherapy of horses. A preliminary clinical report. *Bioelectrochemistry* **2002**, *55(1–2)*, 101–105.

15 Mir, L. M., et al., High-efficiency gene transfer into skeletal muscle mediated by electric pulses. *Proc. Natl. Acad. Sci. USA* **1999**, *96(8)*, 4262–4267.

16 Bureau, M. F., et al., Importance of association between permeabilization and electrophoretic forces for intramuscular DNA electrotransfer. *Biochim. Biophys. Acta* **2000**, *1474(3)*, 353–359.

17 Satkauskas, S., et al., Mechanisms of *in vivo* DNA electrotransfer: respective contributions of cell electropermeabilization and DNA electrophoresis. *Mol. Ther.* **2002**, *5(2)*, 133–140.

18 Paturneau-Jouas, M., et al., Electroporation-mediated delivery of a magnetic resonance imaging contrast agent into muscle to visualize electrotransfer. *Acta Myologica* **2001**, *20*, 174–178.

19 Rols, M. P., et al., Control by ATP and ADP of voltage-induced mammalian-cell-membrane permeabilization, gene transfer and resulting expression. *Eur. J. Biochem.* **1998**, *254(2)*, 382–388.

20 Satkauskas, S., et al., Slow accumulation of plasmid in muscle cells: supporting evidence for a mechanism of DNA uptake by receptor-mediated endocytosis. *Mol. Ther.* **2001**, *4(4)*, 317–323.

21 Cappelletti, M., et al., Gene electro-transfer improves transduction by modifying the fate of intramuscular DNA. *J. Gene Med.* **2003**, *5(4)*, 324–332.

22 Bureau, M. F., et al., Intramuscular plasmid DNA electrotransfer: biodistribution and degradation. *Biochim. Biophys. Acta* **2004**, *1676(2)*, 138–148.

23 Zhang, L., et al., Enhanced delivery of naked DNA to the skin by non-invasive *in vivo* electroporation. *Biochim. Biophys. Acta* **2002**, *1572(1)*, 1–9.

24 Gehl, J., et al., *In vivo* electroporation of skeletal muscle: threshold, efficacy and relation to electric field distribution. *Biochim. Biophys. Acta* **1999**, *1428(2–3)*, 233–240.

25 Miklavcic, D., et al., A validated model of *in vivo* electric field distribution in tissues for electrochemotherapy and for DNA electrotransfer for gene therapy. *Biochim. Biophys. Acta* **2000**, *1523(1)*, 73–83.

26 Rols, M. P., et al., *In vivo* electrically mediated protein and gene transfer in murine melanoma. *Nat. Biotechnol.* **1998**, *16(2)*, 168–171.

27 Mir, L. M., et al., Long-term, high level *in vivo* gene expression after electric pulse-mediated gene transfer into skeletal muscle. *C. R. Acad. Sci. III* **1998**, *321(11)*, 893–899.

28 Heller, R., et al., *In vivo* gene electroinjection and expression in rat liver. *FEBS Lett.* **1996**, *389(3)*, 225–228.

29 Nishi, T., et al., High-efficiency *in vivo* gene transfer using intraarterial plasmid DNA injection following *in vivo* electroporation. *Cancer Res.* **1996**, *56(5)*, 1050–1055.

30 Vicat, J. M., et al., Muscle transfection by electroporation with high-voltage and short-pulse currents provides high-level and long-lasting gene expression. *Hum. Gene Ther.* **2000**, *11(6)*, 909–916.

31 Rubenstrunk, A., Mahfoudi, A., Scherman, D., Delivery of electric pulses for DNA electrotransfer to mouse muscle does not induce the expression of stress related genes. *Cell. Biol. Toxicol.* **2004**, *20(1)*, 25–31.

32 Hartikka, J., et al., Electroporation-facilitated delivery of plasmid DNA in skeletal muscle: plasmid dependence of muscle damage and effect of poloxamer 188. *Mol. Ther.* **2001**, *4(5)*, 407–415.

33 Durieux, A. C., et al., *In vivo* gene electrotransfer into skeletal muscle: effects of plasmid DNA on the occurrence and extent of muscle damage. *J. Gene Med.* **2004**, *6(7)*, 809–816.

34 Gehl, J., Skovsgaard, T., Mir, L. M., Vascular reactions to *in vivo* electroporation: characterization and consequences for drug and gene delivery. *Biochim. Biophys. Acta* **2002**, *1569(1–3)*, 51–58.

35 Bertrand, A., et al., Muscle electrotransfer as a tool for studying muscle fiber-specific and nerve-dependent activity of promoters. *Am. J. Physiol. Cell Physiol.* **2003**, *285(5)*, C1071–C1081.

36 Davis, H. v. L., Whalen, R. G., Demeneix, B. A., Direct gene transfer into skeletal muscle *in vivo*: factors affecting efficiency of transfer and stability of expression. *Hum. Gene Ther.* **1993**, *4(2)*, 151–159.

37 Mennuni, C., et al., Hyaluronidase increases electrogene transfer efficiency in skeletal muscle. *Hum. Gene Ther.* **2002**, *13(3)*, 355–365.

38 Favre, D., et al., Hyaluronidase enhances recombinant adeno-associated virus (rAAV)-mediated gene transfer in the rat skeletal muscle. *Gene Ther.* **2000**, *7(16)*, 1417–1420.

39 McMahon, J. M., et al., Optimisation of electrotransfer of plasmid into skeletal muscle by pretreatment with hyaluronidase – increased expression with reduced muscle damage. *Gene Ther.* **2001**, *8(16)*, 1264–1270.

40 Molnar, M. J., et al., Factors influencing the efficacy, longevity, and safety of electroporation-assisted plasmid-based gene transfer into mouse muscles. *Mol. Ther.* **2004**, *10(3)*, 447–455.

41 Nicol, F., et al., Poly-L-glutamate, an anionic polymer, enhances transgene expression for plasmids delivered by intramuscular injection with *in vivo* electroporation. *Gene Ther.* **2002**, *9(20)*, 1351–1358.

42 Bettan, M., et al., High-level protein secretion into blood circulation after electric pulse-mediated gene transfer into skeletal muscle. *Mol. Ther.* **2000**, *2(3)*, 204–210.

43 Kreiss, P., et al., Erythropoietin secretion and physiological effect in mouse after intramuscular plasmid DNA electrotransfer. *J. Gene Med.* **1999**, *1(4)*, 245–250.

44 Honigman, A., et al., Imaging transgene expression in live animals. *Mol. Ther.* **2001**, *4(3)*, 239–249.

45 Deleuze, V., Scherman, D., Bureau, M. F., Interleukin-10 expression after intramuscular DNA electrotransfer: kinetic studies. *Biochem. Biophys. Res. Commun.* **2002**, *299(1)*, 29–34.

46 Paillard, F., Promoter attenuation in gene therapy: causes and remedies. *Human Gene Therapy* **1997**, *8(17)*, 2009–2010.

47 Aihara, H., Miyazaki. J., Gene transfer into muscle by electroporation *in vivo*. *Nat. Biotechnol.* **1998**, *16(9)*, 867–870.

48 Mathiesen, I., Electropermeabilization of skeletal muscle enhances gene transfer *in vivo*. *Gene Ther.* **1999**, *6(4)*, 508–514.

49 Lu, Q. L., Bou-Gharios, G., Partridge, T. A., Non-viral gene delivery in skeletal muscle: a protein factory. *Gene Ther.* **2003**, *10(2)*, 131–142.

50 Goldspink, G., Skeletal muscle as an artificial endocrine tissue. *Best. Pract. Res. Clin. Endocrinol. Metab.* **2003**, *17(2)*, 211–222.

51 Mir, L. M., et al., Effective treatment of cutaneous and subcutaneous malignant tumours by electrochemotherapy. *Br. J. Cancer* **1998**, *77(12)*, 2336–2342.

52 Mir, L. M., et al., Electrochemotherapy potentiation of antitumour effect of bleomycin by local electric pulses. *Eur. J. Cancer* **1991**, *27(1)*, 68–72.

53 Sersa, G., et al., Electrochemotherapy with cisplatin: potentiation of local cisplatin antitumour effectiveness by application of electric pulses in cancer patients. *Eur. J. Cancer* **1998**, *34(8)*, 1213–1218.

54 Wells, J. M., et al., Electroporation-enhanced gene delivery in mammary tumors. *Gene Ther.* **2000**, *7(7)*, 541–547.

55 Heller, L., et al., Electrically mediated plasmid DNA delivery to hepatocellular carcinomas *in vivo*. *Gene Ther.* **2000**, *7(10)*, 826–829.

56 Bettan, M., et al., Efficient DNA electrotransfer into tumors. *Bioelectrochemistry* **2000**, *52(1)*, 83–90.

57 Fakharzadeh, S. S., et al., Correction of the coagulation defect in hemophilia A mice through factor VIII expression in skin. *Blood* **2000**, *95(9)*, 2799–2805.

58 Jadoul, A., Bouwstra, J., Preat, V. V., Effects of iontophoresis and electroporation on the stratum corneum. Review of the biophysical studies. *Adv. Drug Deliv. Rev.* **1999**, *35(1)*, 89–105.

59 Peachman, K. K., Rao, M., Alving, C. R., Immunization with DNA through the skin. *Methods* **2003**, *31(3)*, 232–242.

60 Drabick, J. J., et al., Cutaneous transfection and immune responses to intradermal nucleic acid vaccination are significantly enhanced by *in vivo* electropermeabilization. *Mol. Ther.* **2001**, *3(2)*, 249–255.

61 Dujardin, N., et al., *In vivo* assessment of skin electroporation using square wave pulses. *J. Control Release* **2002**, *79(1–3)*, 219–227.

62 Herweijer, H., et al., Time course of gene expression after plasmid DNA gene transfer to the liver. *J. Gene Med.* **2001**, *3(3)*, 280–291.

63 Suzuki, T., et al., Direct gene transfer into rat liver cells by *in vivo* electroporation. *FEBS Lett.* **1998**, *425(3)*, 436–440.

64 Liu, F., Huang, L., Electric gene transfer to the liver following systemic administration of plasmid DNA. *Gene Ther.* **2002**, *9(16)*, 1116–1119.

65 Zhang, G., et al., Hydroporation as the mechanism of hydrodynamic delivery. *Gene Ther.* **2004**, *11(8)*, 675–682.

66 Teissie, J., *In vivo* gene expression: combining hydrodynamics-based transfection and electrotransfer. *Trends Biotechnol.* **2002**, *20(12)*, 487–488.

67 Dean, D. A., et al., Electroporation as a method for high-level nonviral gene transfer to the lung. *Gene Ther.* **2003**, *10(18)*, 1608–1615.

68 Matsumoto, T., et al., Successful and optimized *in vivo* gene transfer to rabbit carotid artery mediated by electronic pulse. *Gene Ther.* **2001**, *8(15)*, 1174–1179.

69 Oshima, Y., et al., Targeted gene transfer to corneal stroma *in vivo* by electric pulses. *Exp. Eye Res.* **2002**, *74(2)*, 191–198.

70 Blair-Parks, K., Weston, B. C., Dean, D. A., High-level gene transfer to the cornea using electroporation. *J. Gene Med.* **2002**, *4(1)*, 92–100.

71 Matsuda, T., Cepko, C. L., Electroporation and RNA interference in the rodent retina *in vivo* and *in vitro*. *Proc. Natl. Acad. Sci. USA* **2004**, *101(1)*, 16–22.

72 Mamiya, K., et al., Effects of matrix metalloproteinase-3 gene transfer by electroporation in glaucoma filter surgery. *Exp. Eye Res.* **2004**, *79(3)*, 405–410.

73 Swartz, M., et al., Sparking new frontiers: using *in vivo* electroporation for genetic manipulations. *Dev. Biol.* **2001**, *233(1)*, 13–21.

74 Itasaki, N., Bel-Vialar S., Krumlauf R., 'Shocking' developments in chick embryology: electroporation and in ovo gene expression. *Nat. Cell Biol.* **1999**, *1(8)*, E203–E207.

75 Muramatsu, T., et al., Comparison of three nonviral transfection methods for foreign gene expression in early chicken embryos in ovo. *Biochem. Biophys. Res. Commun.* **1997**, *230(2)*, 376–380.

76 Ohashi, S., et al., Successful genetic transduction *in vivo* into synovium by means of electroporation. *Biochem. Biophys. Res. Commun.* **2002**, *293(5)*, 1530–1535.

77 Grossin, L., et al., Direct gene transfer into rat articular cartilage by *in vivo* electroporation. *Faseb J.* **2003**, *17(8)*, 829–835.

78 Muramatsu, T., et al., Foreign gene expression in the mouse testis by localized *in vivo* gene transfer. *Biochem. Biophys. Res. Commun.* **1997**, *233(1)*, 45–49.

79 Sugihara, K., Park, H. M., Muramatsu, T., *In vivo* gene electroporation confers strong transient expression of foreign genes in the chicken testis. *Poult. Sci.* **2000**, *79(8)*, 1116–1119.

80 Sato, M., et al., Efficient gene delivery into murine ovarian cells by intraovarian injection of plasmid DNA and subsequent *in vivo* electroporation. *Genesis* **2003**, *35(3)*, 169–174.
81 Samakoglu, S., et al., betaMinor-globin messenger RNA accumulation in reticulocytes governs improved erythropoiesis in beta thalassemic mice after erythropoietin complementary DNA electrotransfer in muscles. *Blood* **2001**, *97(8)*, 2213–2220.
82 Payen, E., et al., Improvement of mouse beta-thalassemia by electrotransfer of erythropoietin cDNA. *Exp. Hematol.* **2001**, *29(3)*, 295–300.
83 Maruyama, H., et al., Long-term production of erythropoietin after electroporation-mediated transfer of plasmid DNA into the muscles of normal and uremic rats. *Gene Ther.* **2001**, *8(6)*, 461–468.
84 Adachi, O., et al., Gene transfer of Fc-fusion cytokine by *in vivo* electroporation: application to gene therapy for viral myocarditis. *Gene Ther.* **2002**, *9(9)*, 577–583.
85 Watanabe, K., et al., Protection against autoimmune myocarditis by gene transfer of interleukin-10 by electroporation. *Circulation* **2001**, *104(10)*, 1098–1100.
86 Mallat, Z., et al., Protective role of interleukin-10 in atherosclerosis. *Circ. Res.* **1999**, *85(8)*, e17–e24.
87 Perez, N., et al., Tetracycline transcriptional silencer tightly controls transgene expression after *in vivo* intramuscular electrotransfer: application to interleukin 10 therapy in experimental arthritis. *Hum. Gene Ther.* **2002**, *13(18)*, 2161–2172.
88 Saidenberg-Kermanac'h, N., et al., Efficacy of interleukin-10 gene electrotransfer into skeletal muscle in mice with collagen-induced arthritis. *J. Gene Med.* **2003**, *5(2)*, 164–171.
89 Ho, S. H., et al., Protection against collagen-induced arthritis by electrotransfer of an expression plasmid for the interleukin-4. *Biochem. Biophys. Res. Commun.* **2004**, *321(4)*, 759–766.
90 Kim, J. M., et al., Electro-gene therapy of collagen-induced arthritis by using an expression plasmid for the soluble p75 tumor necrosis factor receptor-Fc fusion protein. *Gene Ther.* **2003**, *10(15)*, 1216–1224.
91 Bloquel, C., et al., Gene therapy of collagen-induced arthritis by electrotransfer of human tumor necrosis factor-alpha soluble receptor I variants. *Hum. Gene Ther.* **2004**, *15(2)*, 189–201.
92 Jeong, J. G., et al., Electrotransfer of human IL-1Ra into skeletal muscles reduces the incidence of murine collagen-induced arthritis. *J. Gene Med.* **2004**, *6(10)*, 1125–1133.
93 Xue, F., et al., Attenuated acute liver injury in mice by naked hepatocyte growth factor gene transfer into skeletal muscle with electroporation. *Gut* **2002**, *50(4)*, 558–562.
94 Prud'homme, G. J., Y. Chang, X. Li, Immunoinhibitory DNA vaccine protects against autoimmune diabetes through cDNA encoding a selective CTLA-4 (CD152) ligand. Hum. *Gene Ther.* **2002**, *13(3)*, 395–406.

95 Long, Y. C., et al., FVIII gene delivery by muscle electroporation corrects murine hemophilia A. *J. Gene Med.* **2004**.

96 Vilquin, J. T., et al., Electrotransfer of naked DNA in the skeletal muscles of animal models of muscular dystrophies. *Gene Ther.* **2001**, *8(14)*, 1097–1107.

97 Murakami, T., et al., Full-length dystrophin cDNA transfer into skeletal muscle of adult mdx mice by electroporation. *Muscle Nerve* **2003**, *27(2)*, 237–241.

98 Gollins, H., et al., High-efficiency plasmid gene transfer into dystrophic muscle. *Gene Ther.* **2003**, *10(6)*, 504–512.

99 Tang, D. C., DeVit, M., Johnston, S. A., Genetic immunization is a simple method for eliciting an immune response. *Nature* **1992**, *356(6365)*, 152–154.

100 Ulmer, J. B., et al., Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* **1993**, *259(5102)*, 1745–1749.

101 van Drunen Littel-van den Hurk, S., Babiuk, S. L., Babiuk, L. A., Strategies for improved formulation and delivery of DNA vaccines to veterinary target species. *Immunol. Rev.* **2004**, *199*, 113–125.

102 Babiuk, L. A., et al., Induction of immune responses by DNA vaccines in large animals. *Vaccine* **2003**, *21(7–8)*, 649–658.

103 Widera, G., et al., Increased DNA vaccine delivery and immunogenicity by electroporation *in vivo*. *J. Immunol.* **2000**, *164(9)*, 4635–4640.

104 Lee, A. H., et al., Comparison of various expression plasmids for the induction of immune response by DNA immunization. *Mol. Cells* **1997**, *7(4)*, 495–501.

105 Kirman, J. R., Seder, R. A., DNA vaccination: the answer to stable, protective T-cell memory? *Curr. Opin. Immunol.* **2003**, *15(4)*, 471–476.

106 Scheerlinck, J. P., et al., *In vivo* electroporation improves immune responses to DNA vaccination in sheep. *Vaccine* **2004**, *22(13–14)*, 1820–1825.

107 Tollefsen, S., et al., Improved cellular and humoral immune responses against Mycobacterium tuberculosis antigens after intramuscular DNA immunisation combined with muscle electroporation. *Vaccine* **2002**, *20(27–28)*, 3370–3378.

108 Bachy, M., et al., Electric pulses increase the immunogenicity of an influenza DNA vaccine injected intramuscularly in the mouse. *Vaccine* **2001**, *19(13–14)*, 1688–1693.

109 Babiuk, S., et al., Electroporation improves the efficacy of DNA vaccines in large animals. *Vaccine* **2002**, *20(27–28)*, 3399–3408.

110 Tollefsen, S., et al., DNA injection in combination with electroporation: a novel method for vaccination of farmed ruminants. *Scand. J. Immunol.* **2003**, *57(3)*, 229–238.

111 Otten, G., et al., Enhancement of DNA vaccine potency in rhesus macaques by electroporation. *Vaccine* **2004**, *22(19)*, 2489–2493.

112 Bakker, J. M., Bleeker, W. K., Parren, P. W., Therapeutic antibody gene transfer: an active approach to passive immunity. *Mol. Ther.* **2004**, *10(3)*, 411–416.

113 Tjelle, T. E., et al., Monoclonal antibodies produced by muscle after plasmid injection and electroporation. *Mol. Ther.* **2004**, *9(3)*, 328–336.

114 Perez, N., et al., Regulatable systemic production of monoclonal antibodies by *in vivo* muscle electroporation. *Genet. Vaccines Ther.* **2004**, *2(1)*, 2.

115 Li, S., et al., Regression of tumors by IFN-alpha electroporation gene therapy and analysis of the responsible genes by cDNA array. *Gene Ther.* **2002**, *9(6)*, 390–397.

116 Lucas, M. L., et al., IL-12 plasmid delivery by *in vivo* electroporation for the successful treatment of established subcutaneous B16.F10 melanoma. *Mol. Ther.* **2002**, *5(6)*, 668–675.

117 Kishida, T., et al., *In vivo* electroporation-mediated transfer of interleukin-12 and interleukin-18 genes induces significant antitumor effects against melanoma in mice. *Gene Ther.* **2001**, *8(16)*, 1234–1240.

118 Tamura, T., et al., Intratumoral delivery of interleukin 12 expression plasmids with *in vivo* electroporation is effective for colon and renal cancer. *Hum. Gene Ther.* **2001**, *12(10)*, 1265–1276.

119 Tamura, T., et al., Combination of IL-12 and IL-18 of electro-gene therapy synergistically inhibits tumor growth. *Anticancer Res.* **2003**, *23(2B)*, 1173–1179.

120 Matsubara, H., et al., Electroporation-mediated transfer of cytokine genes into human esophageal tumors produces anti-tumor effects in mice. *Anticancer Res.* **2001**, *21(4A)*, 2501–2503.

121 Goto, T., et al., Highly efficient electro-gene therapy of solid tumor by using an expression plasmid for the herpes simplex virus thymidine kinase gene. *Proc. Natl. Acad. Sci. USA* **2000**, *97(1)*, 354–359.

122 Shibata, M. A., J. Morimoto, Y. Otsuki, Suppression of murine mammary carcinoma growth and metastasis by HSVtk/GCV gene therapy using *in vivo* electroporation. *Cancer Gene Ther.* **2002**, *9(1)*, 16–27.

123 Goto, T., et al., Combination electro-gene therapy using herpes virus thymidine kinase and interleukin-12 expression plasmids is highly efficient against murine carcinomas *in vivo*. *Mol. Ther.* **2004**, *10(5)*, 929–937.

124 Yamashita, Y., et al., Electroporation-mediated tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)/Apo2L gene therapy for hepatocellular carcinoma. *Hum. Gene Ther.* **2002**, *13(2)*, 275–286.

125 Celiker, M. Y., et al., Inhibition of Wilms' tumor growth by intramuscular administration of tissue inhibitor of metalloproteinases-4 plasmid DNA. *Oncogene* **2001**, *20(32)*, 4337–4343.

126 Baba, M., Iishi, H., Tatsuta, M., Transfer of bcl-xs plasmid is effective in preventing and inhibiting rat hepatocellular carcinoma induced by N-nitrosomorpholine. *Gene Ther.* **2001**, *8(15)*, 1149–1156.

127 Bossard, C., et al., Antiangiogenic properties of fibstatin, an extracellular FGF-2-binding polypeptide. *Cancer Res.* **2004**, *64(20)*, 7507–7512.

128 Razin, A., Riggs, A. D., DNA methylation and gene function. *Science* **1980**, *210(4470)*, 604–610.

129 Slack, A., et al., Antisense MBD2 gene therapy inhibits tumorigenesis. *J. Gene Med.* **2002**, *4(4)*, 381–389.

130 Ivanov, M. A., et al., Enhanced antitumor activity of a combination of MBD2-antisense electrotransfer gene therapy and bleomycin electrochemotherapy. *J. Gene Med.* **2003**, *5(10)*, 893–899.
131 Cemazar, M., et al., Effective gene transfer to solid tumors using different nonviral gene delivery techniques: electroporation, liposomes, and integrin-targeted vector. *Cancer Gene Ther.* **2002**, *9(4)*, 399–406.
132 Inoue, T., Krumlauf, R., An impulse to the brain – using *in vivo* electroporation. *Nat. Neurosci.* **2001**, 4 Suppl., 1156–1158.
133 Saito, T., Nakatsuji, N., Efficient gene transfer into the embryonic mouse brain using *in vivo* electroporation. *Dev. Biol.* **2001**, *240(1)*, 237–246.
134 Tawk, M., et al., High-efficiency gene transfer into adult fish: a new tool to study fin regeneration. *Genesis* **2002**, *32(1)*, 27–31.
135 Haas, K., et al., Targeted electroporation in Xenopus tadpoles *in vivo* – from single cells to the entire brain. *Differentiation* **2002**, *70(4–5)*, 148–154.
136 Konig, S., et al., Modular organization of phylogenetically conserved domains controlling developmental regulation of the human skeletal myosin heavy chain gene family. *J. Biol. Chem.* **2002**, *277(31)*, 27593–27605.
137 Lamartina, S., et al., Stringent control of gene expression *in vivo* by using novel doxycycline-dependent trans-activators. *Hum. Gene Ther.* **2002**, *13(2)*, 199–210.
138 Payen, E., et al., Oxygen tension and a pharmacological switch in the regulation of transgene expression for gene therapy. *J. Gene Med.* **2001**, *3(5)*, 498–504.
139 Trollet, C., et al., Regulation of gene expression using a conditional RNA antisense strategy. *J. Genome Sci. Tech.* **2004**, *3*, 1–13.
140 Rao, M., et al., *In vivo* comparative study of RNAi methodologies by in ovo electroporation in the chick embryo. *Dev. Dyn.* **2004**, *231(3)*, 592–600.
141 Pekarik, V., et al., Screening for gene function in chicken embryo using RNAi and electroporation. *Nat. Biotechnol.* **2003**, *21(1)*, 93–96.
142 Konishi, Y., et al., Cdh1-APC controls axonal growth and patterning in the mammalian brain. *Science* **2004**, *303(5660)*, 1026–1030.
143 Kishida, T., et al., Sequence-specific gene silencing in murine muscle induced by electroporation-mediated transfer of short interfering RNA. *J. Gene Med.* **2004**, *6(1)*, 105–110.
144 Krull, C. E., A primer on using in ovo electroporation to analyze gene function. *Dev. Dyn.* **2004**, *229(3)*, 433–439.

12
Electrogenetransfer in Clinical Applications

*Lluis M. Mir**

12.1
Summary of the Basis of Electrogenetherapy

The electrical pulses in DNA electrotransfer have two roles: the "electroporation" of the target cells and the electrophoretic transport of the DNA "towards or across" the cell membrane.

12.1.1
Tissue Electropermeabilization

The application of cell electropermeabilization (also termed cell electroporation) to DNA transfer to living cells was first described, *in vitro*, by E. Neumann in 1982 [1]. Its development to the efficient and safe use of electrical pulses *in vitro*, and later on *in vivo*, took a number of years during which several methods to analyze cell

* In the name of the CLINIPORATOR and ESOPE consortia: F. André[1)], N. Bédaïria[2)], E. Bergamini[3)], C. Bertacchini[3)], V. Billard[2)], R. Cadossi[3)], M. Cemazar[4)], B. Christiansen[5)], D. Cukjati[6)], C. Collins[7)], C. Cournil-Henrionnet[1)], C. Cronin[7)], C. Dunne[7)], J. Eriksen[5)], K. Flisar[6)], B. Gabriel[8], J.-R. Garbay[2)], P. Geertsen[5)], J. Gehl[5)], M. Golzio[8)], T. Jarm[6)], S. Kakorin[9)], I. Krøyer-Kaiser[5)], M. Friis-Knudsen[5)], T. Kotnik[6)], P. Kramar[6)], J. Larkin[7)], V. Leben[6)], P.-M. Margotti[3)], M. Marty[2)], D. Miklavcic[6)], L. M. Mir[1)], N. Morsli[2)], E. Neumann[9)], G. O'Sullivan[7)], M. Pavlin[6), 9)], I. Pavlovic[6)], N. Pavsel[6), 10)], U. Pliquett[9)], M. Puc[6)], V. Préat[10)], C. Robert[2)], M. P. Rols[8)], Z. Rudolf[4)], S. Sagouati[2)], P. H. Moller[5), 8)], S. Satkauskas[1), 11)], M. Schmeer[9)], D. Sel[6)], G. Selvaggi[3)], D. Semrov[6)], G. Sersa[4)], B. Slekovec[4)], D. Soden[7)], J. Teissié[8)], K. Toensing[9)], S. Wehmeyer[5)].

1) UMR 8121 CNRS – Institut Gustave-Roussy, Villejuif, France
2) Institut Gustave-Roussy, Villejuif, France
3) IGEA S.r.l., Carpi (MO), Italy
4) Institute of Oncology, Ljubljana, Slovenia
5) Department of Oncology, Herlev Hospital, Herlev, Denmark
6) Faculty of Electrical Engineering, Ljubljana, Slovenia
7) Cork University Cancer Center, Cork, Ireland
8) UMR 5089, IPBS du CNRS, Toulouse, France
9) Faculty of Chemistry, University of Bielefeld, Bielefeld, Germany
10) Unité de Pharmacie Galénique, Université Catholique de Louvain, Brussels, Belgium
11) Present address: Department of Biology, University of Kaunas, Kaunas, Lithuania

electropermeabilization were developed. It was thus demonstrated, through the use of trains of identical electrical pulses, that cell permeabilization, allowing highly efficient DNA electrotransfer, does indeed occur *in vivo* under electrical conditions [2–4]. However, the initial studies on gene electrotransfer had already shown that long pulses of 5 to 50 ms duration [2, 5–7] were more efficient than short pulses of 100 μs and that the electropermeabilization of the cells in the tissue alone was not sufficient. *In vivo*, cells remain permeabilized for quite a long period (several minutes) [8–10], but DNA has to be injected before the delivery of the pulse. Later on we actually demonstrated that the electropermeabilization, while essential, is not the only effect of the electrical pulses.

12.1.2
DNA Electrophoresis

Provided that the voltage used is not detrimental to tissue preservation (excessive electric field strengths may result in the irreversible permeabilization of the cells or in supraphysiological heating of the tissue, particularly with long pulses), the efficacy of electrotransfer depends mainly on the duration of the electrical pulses. During long pulses, DNA electrophoresis can take place within a tumor, the electrophoretic forces allowing the DNA to be transported from the bulk of the liquid injected into the tissue to the vicinity of the cell membranes, facilitating the interaction of the DNA with the electropermeabilized membranes and finally the uptake of the DNA by the cells.

12.1.3
The Interest of Electrogenetherapy

First of all, DNA electrotransfer is a nonviral approach, and as such is much safer than viral approaches. Safety is now the first concern for gene therapy development, and while viral systems are potentially very efficient, two factors – safety and ease of manufacturing – suggest that nonviral gene delivery systems will be the preferred choice in the future. The simplest, least toxic product that can be envisaged is the naked DNA itself. Appropriate DNA constructs can produce high expression levels and simultaneously avoid the dangers of producing recombinant virus or other toxic effects engendered by biologically active viral particles. Also, the manufacture of naked DNA is less complex than the use of tissue culture cells as bioreactors for virus production, and QA/QC procedures should be simpler.

DNA electrotransfer is interesting because it is based on cell electropermeabilization, a physical means of perturbation of the structure of the cell membrane and hence its functional impermeability to hydrophilic substances. With the use of electrical pulses there is no addition or removal of membrane components, as is the case when chemical means of permeabilization are used, so full recovery is facilitated. Moreover, under appropriate conditions, cell electropermeabilization is a nonthermal effect and does not provoke protein denaturation (which also facilitates cell recovery). Safety can therefore be as good as possible, provided that appropriate electrical parameters are applied.

The other point of interest of the method is that it is simple, since it only requires the DNA to be injected into the tissue and the electrical pulses to be delivered. The main interest of *in vivo* DNA electrotransfer, however, is that it allows the efficient transfer of genes into tissues, although the method is restricted, *in vivo*, to solid tissues accessible to the electrodes. DNA electrotransfer is so far the most general and most efficient method for physical transfer of DNA into target cells *in vivo*, all other methods suffering greater restrictions: biolistic approaches (see Chapter 9) are limited to surface tissues, the hydrodynamic method (see also Chapter 10) is essentially efficient only for DNA transfer to liver cells, and DNA injection is much less efficient. Moreover, no chemical method works better *in vivo* than the direct electrotransfer of naked DNA. The method is also very rapid; new constructs made by conventional molecular biology approaches can be amplified by rapid "mini-preparations" of DNA and quantified by optical density determination, after which it is sufficient merely to adjust plasmid concentration, to inject it into the tissues, and to expose the tissue to appropriate electrical pulses (with viral methods, constructs must be inserted in a viral backbone ground and transfected in engineered producing cells, and the viruses must then be produced, collected, isolated, concentrated, and titrated before injection).

12.2
The Road to Clinical Electrogenetherapy

12.2.1
Basic Difficulties and Requirements

12.2.1.1 Electrogenetherapy is a Local Treatment

EGT can only deliver local gene transfer and DNA expression: only tissues(s) covered by the electric fields, at appropriate field strengths, become susceptible (electropermeabilized) to DNA uptake. Similarly, the electrical pulses exert their electrophoretic effects only in approximately the same volume. EGT will therefore be ineffective (at least *in vivo*) in any tissue not composed of firm solid masses. Cells such as white blood cells, however, can be efficiently electrotransfected *ex vivo* and then reinjected into the body [11]. Continuous flow devices for the treatment of large volumes of cells have been developed [11, 12].

As a local treatment, EGT is also best suited for gene transfer to organs with clear physical borders, though the overall volume of tissue covered by the electrodes may be larger than the target tissue (that is, other neighboring tissues may also be between the electrodes). Safety is maintained on the one hand because the pulses only reversibly permeabilize the cells in the tissues and should not damage the exposed tissues, while the selectivity of the transfer, on the other hand, should be guaranteed by the DNA injection itself.

12.2.1.2 DNA Injection

DNA injection is a crucial step, since DNA electrotransfer can only occur in the organ/tissue (or part of it) in which the DNA-containing fluid distributes after injection. The DNA thus generally has to be injected locally, since intravenous injection would result in an extremely large dilution of the DNA, without any guarantee that it would cross the vascular endothelial barrier within the target tissue to arrive close to the target cells. The intravenous route would be valid only if associated with high liquid pressure, as has been demonstrated in rodent liver [hydrodynamic DNA transfer, after the very rapid injection of the DNA (5 seconds) in a very large volume (10% of the body weight) through the tail vein] or in the case of the skeletal muscle (after clamping of all the efferent vessels). In these cases the pressure may help both in crossing the endothelial barrier and, to some extent, in entering the cells.

12.2.1.3 Need for Appropriate Electrodes

Electrodes adapted to the tissue are required and are still the reason for the existence of a large research effort demanding the combined skills of biologists trained in anatomy and physiology and of biomedical engineers to help in defining optimal field distributions within tissues. Particularly important is the relative reduction of the field intensity close to the electrodes, as well as the reduction of electrochemical reactions at the electrode surfaces. A first option is to use electrodes that divide the volume to be treated into small unitary volumes requiring lower voltages (lower electric field strengths) [13], although this volume division increases the number of pulses needed to treat the whole volume of the target tissue.

12.2.1.4 Need for Appropriate Electrical Pulse Generators

Pulse generators have to be able to deliver both high voltage and high amperages, in a highly controllable fashion. This means particular devices designed specially. Moreover, they also have to comply to the strictest safety rules (satisfying the CE marking, for example).

12.2.1.5 Electrogenetherapy and Public and Professional Perceptions of the Biomedical Use of Electricity

Even though electricity is everywhere in our environment, its direct use in biomedicine is not yet very popular. Some applications – iontophoresis for the transdermal delivery of drugs, for example – have entered everyday use, but unfortunate Frankenstein connotations persist, particularly in the case of EGT, in which the intensity of the pulses provokes contraction of the muscles treated (or of muscles lying very close to the target organ) and unsettling sensations (even pain, if the sensations are intense). However, the simplicity, the safety, and the efficacy of the EGT provide quite a high benefit to risk ratio, which is a great argument for EGT development. Nevertheless, for acceptance of EGT, the concepts of electrogenetherapy need to become familiar not only to health care professionals but also to the general public and other stakeholders.

12.2.2
The CLINIPORATOR Project

In 1999 the European Commission funded the CLINIPORATOR project (QLK3-1999-00484, **CLINIPORATOR**: a new adaptive generator for DNA electrotransfer *in vivo* for gene therapy). This project has allowed us to demonstrate the basis of the efficacy of EGT. We have shown that cell permeabilization, or at least some sort of membrane destabilization, is indispensable with, but that the efficacy of the EGT is essentially a function of the quality of the electrophoretic component of the electrical pulses. We have also developed models for the progression of cell permeabilization within the target tissues [14, 15] and have analyzed the electrical behavior of cells [16, 17] and of tissues [18, 19]. We have demonstrated the interest of combinations of high voltage, short (100 μs) pulses (HV pulses) and of lower voltage, long (100 ms) pulses (LV pulses) [9] and have developed a new generator (the Cliniporator™) based on the use of HV + LV combinations. In this context it is important to stress that the contribution of the HV pulse is to permeabilize the cells while that of the LV is the electrophoretic transport of the DNA, and also that we have developed an algorithm for the online (real time) control of the HV pulse voltage. If the algorithm-based device detects that the pulse will be too intense and thus detrimental to tissue viability, then pulse voltage is corrected shortly after the beginning of the pulse, before the set (too high) voltage value is reached, preventing the delivery of an excessive voltage. Therefore, the detrimental voltage value is not reached and the pulse stays safe and efficient (D. Cukjati et al., in preparation).

Finally, and not least, the Cliniporator™:

- has a friendly user interface, setting the recommended electrical parameters as a function of the electrodes used, but also providing the user with complete freedom to choose other parameters;
- displays the curves of the voltage applied and of the current delivered during the pulse on a screen after each application;
- possesses data storage capabilities, as a function of the patient, the session, and the application;
- stores all the voltage and current curves in these organized data storage capabilities, and these data can be easily exported; and
- has received the CE mark.

These features, and other information (specialized courses, meetings, consortium newsletters, recent publications) can be found at the project website: www.cliniporator.com

12.2.3
The ESOPE Project

In 2002 the European Commission funded the project ESOPE (QLK3-2002-02003: ESOPE: European Standard Operating Procedures for Electrochemotherapy and Electrogenetherapy). This project has already allowed us to test the Cliniporator™

device in a clinical setting for another application of the permeabilizing electrical pulses: namely electrochemotherapy (ECT).

ECT is a new antitumor approach based on the combination of permeabilizing electrical pulses and anticancer drugs that do not enter the cells by diffusion through the plasma membrane (nonpermeant drugs such as bleomycin) or that do not freely cross the cell membrane (poorly permeant drugs such as cisplatin). ECT has shown great potential for the treatment of solid tumors, and many preclinical and clinical studies have reported its efficacy in a large variety of tumors. In ECT the only effect of the electrical pulses is the permeabilization of the target cells (which has to be as close to optimal as possible). There is no need for electrophoretic transport of the cytotoxic compounds, since anticancer agents are very small molecules in relation to normal plasmids of several kb (one kb equates roughly to 600 000 Daltons, while bleomycin, one of the largest anticancer drugs, has a molecular weight of only 1500 Daltons). Once the cells are permeabilized, the anticancer agents then enter the cells by simple diffusion (very high diffusion coefficients of DNA molecules prevent such a simple uptake mechanism). Thus, for ECT, HV delivery is sufficient.

The Cliniporator™ has already been used for the treatment of tumor nodules in 59 patients with good ECT clinical results (not yet reported, ongoing study). Importantly, the project has allowed the best approaches for limiting the disagreeable sensations linked to the HV delivery to be defined as a function of the size and localization of the treated lesions. The safe and appropriate use of the Cliniporator™ device in clinics for the delivery of the HV pulses has thus been demonstrated, and standard operating procedures for the use of the HV pulses have been written and are presently being submitted to validation.

The way is thus prepared for clinical trials involving the injection of GMP DNA (see also Chapter 3) and combinations of HV + LV pulses for the clinical implementation of electrogenetherapy (DNA electrotransfer).

12.2.4
Future Perspectives

As concluded above, the preliminary steps for EGT trials have been covered. The equipment to bring EGT to the clinical stage is ready. Nevertheless, it is clear that there is still room for further improvement in electrode design and adaptation to specific tissues. Of course, it will be necessary to define the indications of this nonviral method, precisely taking into account that the two main predictable targets of EGT (at least in the initial steps of EGT development) will be skeletal muscle and tumors. Both tissue types can be easily accessed and both present evident interest:

- Tumor growth is still a clinical problem and new approaches must be undertaken, particularly for applications – such as the use of genes coding for immunostimulating factors – that have already been validated in preclinical studies [20, 21].

- Skeletal muscle is a convenient cell factory for the production either of secreted factors acting in a systemic way, to correct metabolic diseases [22–24], for example, or of secreted factors that will act locally at distant places, including DNA vaccination (see Chapter 6) [25–30]. Indeed, muscle is a very good secreting organ and it is important to recall that the electrotransfer can be repeated [31], that sustained expression for at least nine months has been shown after gene electrotransfer to skeletal muscle [6], and that several plasmids can be coelectrotransferred to the same muscle fibers [32].

References

1 Neumann, E., Schaefer-Ridder, M., Wang, Y., Hofschneider, P. H., *Embo J.* **1982**, *1*, 841–845.
2 Rols, M. P., Delteil, C., Golzio, M., Dumond, P., Cros, S., Teissie, J., *Nat. Biotechnol.* **1998**, *16*, 168–171.
3 Mir, L. M., Bureau, M. F., Gehl, J., Rangara, R., Rouy, D., Caillaud, J. M., Delaere, P., Branellec, D., Schwartz, B., Scherman, D., *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 4262–4267.
4 Gehl, J., Mir, L. M., *Biochem. Biophys. Res. Commun.* **1999**, *261*, 377–380.
5 Suzuki, T., Shin, B. C., Fujikura, K., Matsuzaki, T., Takata, K., *FEBS Lett.* **1998**, *425*, 436–440.
6 Mir, L. M., Bureau, M. F., Rangara, R., Schwartz, B., Scherman, D., *C. R. Acad. Sci. III* **1998**, *321*, 893–899.
7 Aihara, H., Miyazaki, J., *Nat. Biotechnol.* **1998**, *16*, 867–870.
8 Gehl, J., Skovsgaard, T., Mir, L. M., *Biochim. Biophys. Acta* **2002**, *1569*, 51–58.
9 Satkauskas, S., Bureau, M. F., Puc, M., Mahfoudi, A., Scherman, D., Miklavcic, D., Mir, L. M., *Mol. Ther.* **2002**, *5*, 133–140.
10 Sersa, G., Krzic, M., Sentjurc, M., Ivanusa, T., Beravs, K., Kotnik, V., Coer, A., Swartz, H. M., Cemazar, M., *Br. J. Cancer* **2002**, *87*, 1047–1054.
11 Sixou, S., Teissie, J., *Biochem. Biophys. Res. Commun.* **1992**, *186*, 860–866.
12 Zeira, M., Tosi, P. F., Mouneimne, Y., Lazarte, J., Sneed, L., Volsky, D. J., Nicolau, C., *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 4409–4413.
13 Ramirez, L. H., Orlowski, S., An, D., Bindoula, G., Dzodic, R., Ardouin, P., Bognel, C., Belehradek, J., Jr., Munck, J. N., Mir, L. M., *Br. J. Cancer* **1998**, *77*, 2104–2111.
14 Sel, D., Cukjati, D., Batiuskaite, D., Slivnik, T., Mir, L. M., Miklavcic, D., *IEEE Trans. Biomed. Eng.* **2005**, *52*, 816–827.
15 Pavselj, N., Bregar, Z., Cukjati, D., Batiuskaite, D., Mir, L. M., Miklavcic, D., *IEEE Trans. Biomed. Eng.*, in press.
16 Kotnik, T., Miklavcic, D., *Biophys. J.* **2000**, *79*, 670–679.
17 Kotnik, T., Pucihar, G., Rebersek, M., Miklavcic, D., Mir, L. M., *Biochim. Biophys. Acta* **2003**, *1614*, 193–200.
18 Pliquett, U., Elez, R., Piiper, A., Neumann, E., *Bioelectrochemistry* **2004**, *62*, 83–93.

19 Cima, L. F., Mir, L. M., *Appl. Phys. Lett.* **2004**, *85*, 4520–4522.

20 Heller, L., Pottinger, C., Jaroszeski, M. J., Gilbert, R., Heller, R., *Melanoma Res.* **2000**, *10*, 577–583.

21 Heller, L. C., Ingram, S. F., Lucas, M. L., Gilbert, R. A., Heller, R., *Technol. Cancer Res. Treat.* **2002**, *1*, 205–209.

22 Kreiss, P., Bettan, M., Crouzet, J., Scherman, D., *J. Gene Med.* **1999**, *1*, 245–250.

23 Lamartina, S., Roscilli, G., Rinaudo, C. D., Sporeno, E., Silvi, L., Hillen, W., Bujard, H., Cortese, R., Ciliberto, G., Toniatti, C., *Hum. Gene Ther.* **2002**, *13*, 199–210.

24 Rizzuto, G., Cappelletti, M., Mennuni, C., Wiznerowicz, M., DeMartis, A., Maione, D., Ciliberto, G., La Monica, N., Fattori, E., *Hum. Gene Ther.* **2000**, *11*, 1891–1900.

25 Babiuk, S., Baca-Estrada, M. E., Foldvari, M., Storms, M., Rabussay, D., Widera, G., Babiuk, L. A., *Vaccine* **2002**, *20*, 3399–3408.

26 Peretz, Y., Zhou, Z. F., Halwani, F., Prud'homme, G. J., *Mol. Ther.* **2002**, *6*, 407–414.

27 Wu, C. J., Lee, S. C., Huang, H. W., Tao, M. H., *Vaccine* **2004**, *22*, 1457–1464.

28 Perez, N., Plence, P., Millet, V., Greuet, D., Minot, C., Noel, D., Danos, O., Jorgensen, C., Apparailly, F., *Hum. Gene Ther.* **2002**, *13*, 2161–2172.

29 Saidenberg-Kermanac'h, N., Bessis, N., Deleuze, V., Bloquel, C., Bureau, M., Scherman, D., Boissier, M. C., *J. Gene Med.* **2003**, *5*, 164–171.

30 Draghia-Akli, R., Ellis, K. M., Hill, L. A., Malone, P. B., Fiorotto, M. L., *Faseb J.* **2003**, *17*, 526–528.

31 Rizzuto, G., Cappelletti, M., Maione, D., Savino, R., Lazzaro, D., Costa, P., Mathiesen, I., Cortese, R., Ciliberto, G., Laufer, R., La Monica, N., Fattori, E., *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 6417–6422.

32 Martel-Renoir, D., Trochon-Joseph, V., Galaup, A., Bouquet, C., Griscelli, F., Opolon, P., Opolon, D., Connault, E., Mir, L., Perricaudet, M., *Mol. Ther.* **2003**, *8*, 425–433.

13
Cancer Inhibition in Mice After Systemic Application of Plasmid-Driven Expression of Small Interfering RNAs

Birgit Spänkuch and Klaus Strebhardt

13.1
Introduction

RNA interference is an excellent strategy for gene silencing *in vitro* (Hannon, 2002; McManus et al., 2002; Tuschl, 2002). Tuschl and colleagues showed that transfection of synthetic 21-nucleotide small interfering RNA (siRNA) duplexes into mammalian cells efficiently inhibits endogenous gene expression in a sequence-specific manner (Elbashir et al., 2001). However, phenotypic changes induced by siRNAs persist for at most a week in cell culture, which limits their utility. The main obstacle to achieve gene silencing by siRNAs in animals is delivery. Downregulation of gene expression in mice through the use of high-pressure, high-volume, intravenous (i.v.) injection of synthetic siRNAs has been demonstrated in an investigation of whether i.v. siRNA injection targeting Fas would be able to inhibit Fas expression in mouse hepatocytes *in vivo* and protect the liver from fulminant hepatitis and fibrosis (Song et al., 2003). In hepatitis induced by injection of agonistic Fas-specific antibodies, 82% of mice treated with siRNA that efficiently silenced Fas survived for 10 days of observation, whereas control mice died within three days. Silencing of Fas expression with RNAi holds therapeutic promise for prevention of liver injury by protecting hepatocytes from cytotoxicity. Alternative approaches use viral vector delivery for the expression of small hairpin RNAs to achieve RNAi-based gene silencing. The dominant polyglutamine expansion diseases, which include spinocerebellar ataxia type 1 (SCA1) and Huntington disease, are progressive, untreatable, neurodegenerative disorders. Upon intracerebellar injection, recombinant adeno-associated virus vector expression of short hairpin RNAs targeted to the mutant allele profoundly improved motor coordination and restored cerebellar morphology (Xia et al., 2004). Although both experimental approaches (high-pressure, high-volume i.v. injection and virus-mediated delivery) seem to demonstrate the potential use of RNAi *in vivo* as therapy for human diseases, both strategies have limited if any clinical use due to safety concerns and high-risk side effects. For these reasons, alternative methods that reduce potential risks for future clinical trails are of utmost interest. The use of short hairpin RNAs (shRNAs) driven by polymerase III promoters integrated into

bacterial plasmids is currently under investigation as an alternative strategy to suppress undesirable gene expression more safely and stably. Such constructs, with well defined initiation and termination sites, have been used to produce various small RNA species that inhibit the expression of genes with diverse functions in mammalian cell lines (Lobo et al., 1990; Hannon et al., 1991; Lee et al., 2002; Chong et al., 2001; Paul et al., 2002; Sui et al., 2002). A novel approach using a nonviral vector for the expression of small hairpin RNAs in mice is discussed in this article.

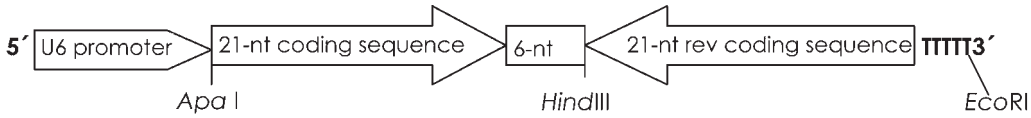
RNA interference has been used to investigate the role of the polo-like kinase 1 (PLK1) protein in neoplastic proliferation (Spankuch-Schmitt et al., 2002). PLK1 is a serine/threonine kinase that is highly conserved between yeasts and humans and plays an important role in cell cycle regulation (Glover et al., 1998). PLK1 expression is elevated in neoplastic tissues and may be a potential prognostic factor for many human cancers (Strebhardt, 2001). All cancer cell lines (MCF-7 breast, HeLa S3 cervical, SW-480 colon, and A549 lung cancer cells) transfected with low doses of siRNAs targeted towards PLK1 had greatly decreased levels of PLK1 mRNA and protein relative to those in corresponding cells transfected with scrambled control siRNAs (Spankuch-Schmitt et al., 2002). Downregulation of PLK1 expression by siRNA administration induced apoptosis in various types of cancer cells. Primary human mammary epithelial cells take up siRNAs less efficiently than cancer cells do, however, and transfection of such cells with PLK1 siRNAs slowed their proliferation only transiently (Spankuch-Schmitt et al., 2002). In view of the differential effect of siRNA targeted towards PLK1 in tumor cells versus that in normal proliferating cells, PLK1 is likely to be a challenging target for tumor therapy.

In vivo delivery of siRNAs has been shown to inhibit transgene expression in certain organs, predominately the liver, in adult mice (Brummelkamp et al., 2002; Lewis et al., 2002; McCaffrey et al., 2002; Xia et al., 2002). Inhibition of tumor cell proliferation by systemic treatment of tumor-bearing animals with siRNAs has not, to the best of our knowledge, been demonstrated previously but is a potentially important therapeutic strategy because metastasis is the main cause of treatment failure and death from cancer. Unmodified siRNAs are unlikely to cause longlasting changes, however, so investigations have been made into whether transfection of cancer cells with plasmids expressing shRNAs targeted towards human PLK1 and driven by a human U6 promoter would inhibit the expression of PLK1 mRNA and protein in cell culture and whether intravenous injection of such plasmids into tumor-bearing mice would suppress PLK1 expression and tumor growth.

13.2
Plasmid-Expressed siRNA

13.2.1
PLK1 shRNA-Mediated Inhibition of PLK1 Expression

DNA constructs (pBS/U6/shRNA/PLK1 and pBS/U6/shRNA/PLK1S, respectively) for the synthesis of shRNAs corresponding to the recently described siRNA2 –



shRNA/PLK1 GGGCGGCUUUGCCAAGUGCUU
UUUUCCCGCCGAAACGGUUCACGAA

shRNA/PLK1S GGGCCCUGUACUAGGUUGCUG
UUUUCCCGGGACAUGAUCCAACGAC

Figure 13.1 Strategy for generating short hairpin RNA (shRNA) specific for polo-like kinase 1 (PLK1). An inverted repeat was inserted at position +1 of the U6 promoter (positions –315 to +1). The specific motif is 21 nucleotides (nt) long and corresponds to the coding region of the PLK1 gene. The two sequences forming the inverted repeat are separated by a 6-nt spacer. A transcription termination signal for RNA polymerase III containing five thymidine residues is attached to the 3′-end of the inverted repeat. The parental plasmid, without the inverted repeat, is pBS/U6. DNA sequences for pBS/U6/shRNA/PLK1 and for the scrambled control pBS/U6/shRNA/PLK1S are shown.

PLK1 shRNA, which efficiently inhibits PLK1 expression in HeLa S3 cells, and PLK1S shRNA, the scrambled version of siRNA2, which did not inhibit PLK1 expression – were generated (Spankuch-Schmitt et al., 2002). Each construct produced an shRNA composed of two 21-nucleotide PLK1 sequences in an inverted orientation to each other, separated by a six-nucleotide spacer, and each construct also had a 3′ RNA polymerase III termination signal sequence of five thymidine residues (Figure 13.1).

Northern blot analysis was used to investigate whether transfection of HeLa S3 cells with PLK1 shRNA or PLK1S shRNA constructs would alter the level of PLK1 mRNA in relation to that in cells transfected with the control parental plasmid pBS/U6. The level of PLK1 mRNA in cells expressing PLK1 shRNA was statistically significantly lower than that in cells expressing the control scrambled PLK1S shRNA or the parental vector, at all plasmid concentrations tested (50% reduction relative to control cells; $P = 0.04$). Western blot analysis was used to determine whether the reduced levels of PLK1 mRNA observed after PLK1 shRNA transfection also reflected reduced PLK1 protein expression (Figure 13.2). The levels of PLK1 protein in cells transfected 96 hours earlier with PLK1 shRNA vectors were significantly lower than those in cells transfected with the parental vector or the scrambled PLK1S shRNA vector at all plasmid concentrations tested (75% reduction relative to control cells, $P = 0.01$). Furthermore, depletion of cells of PLK1 by PLK1 shRNA vector transfection also affected cancer cell proliferation. Whilst the proliferation of cells transfected with the parental vector or with the scrambled control PLK1S shRNA vector was not altered relative to untreated cells, proliferation of HeLa S3 cells

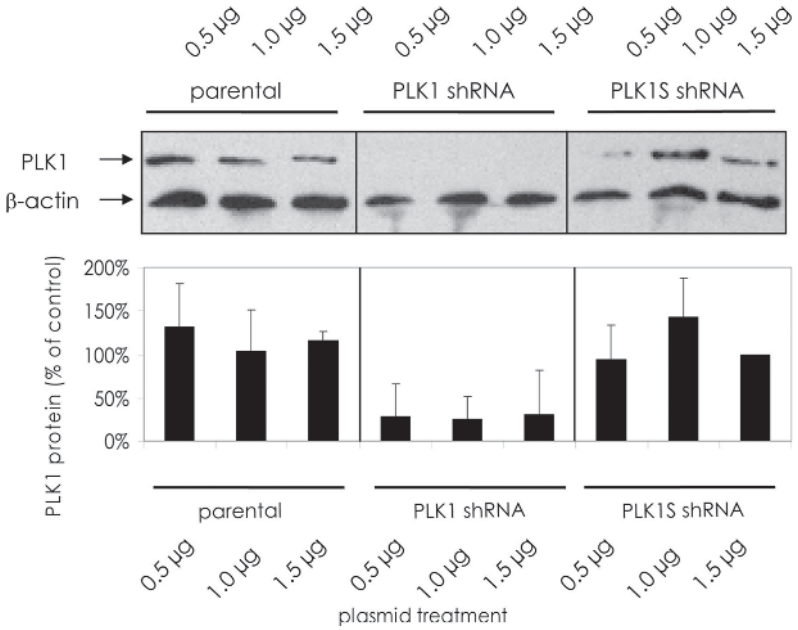


Figure 13.2 Western blot analysis of PLK1-specific (PLK1 = polo-like kinase 1) short hairpin RNAs (shRNAs) and PLK1 expression in transfected cultured HeLa S3 cervical cancer cells. Cells were transfected with a combination of recombinant plasmids, as indicated, and pPuro, a plasmid carrying the gene for puromycin resistance (ratio = 1 pPuro/10 recombinant plasmids). To control for variability of loading, membranes were reexamined with antibodies against β-actin.

transfected with the PLK1 shRNA plasmid was reduced by 89% ($P = 0.04$) relative to the scrambled control.

13.2.2
Nuclease Inhibitor ATA and Stability of Plasmid DNA in Mammalian Blood

A potential barrier to the successful transfection of foreign DNA into mammalian cells *in vivo* is the activity of various bloodborne nucleases. To protect plasmids from the nucleases in peripheral blood from nude mice it is possible to use ATA (aurintricarboxylic acid), which inhibits DNase I, RNase A, S1 nuclease, exonuclease III, and various endonucleases (Blumenthal et al., 1973; Hallick et al., 1977) in *ex vivo* plasmid degradation assays. The mass of DNA, the volume of peripheral blood, and the incubation temperature were kept constant, but incubation time varied. Plasmid integrity was assessed by Southern blot analysis.

When pure plasmid DNA (PLK1 shRNA) was incubated in murine blood, most supercoiled plasmid (CCC) had disappeared by 30 minutes, and the corresponding degradation products (circular [OC] and linear [L] forms) were detectable for up to four hours (Figure 13.3 A and B). If plasmids were first mixed with ATA at weight ratios of DNA to ATA of 50 : 1, 5 : 1, or 0.5 : 1 and the mixture was then added to

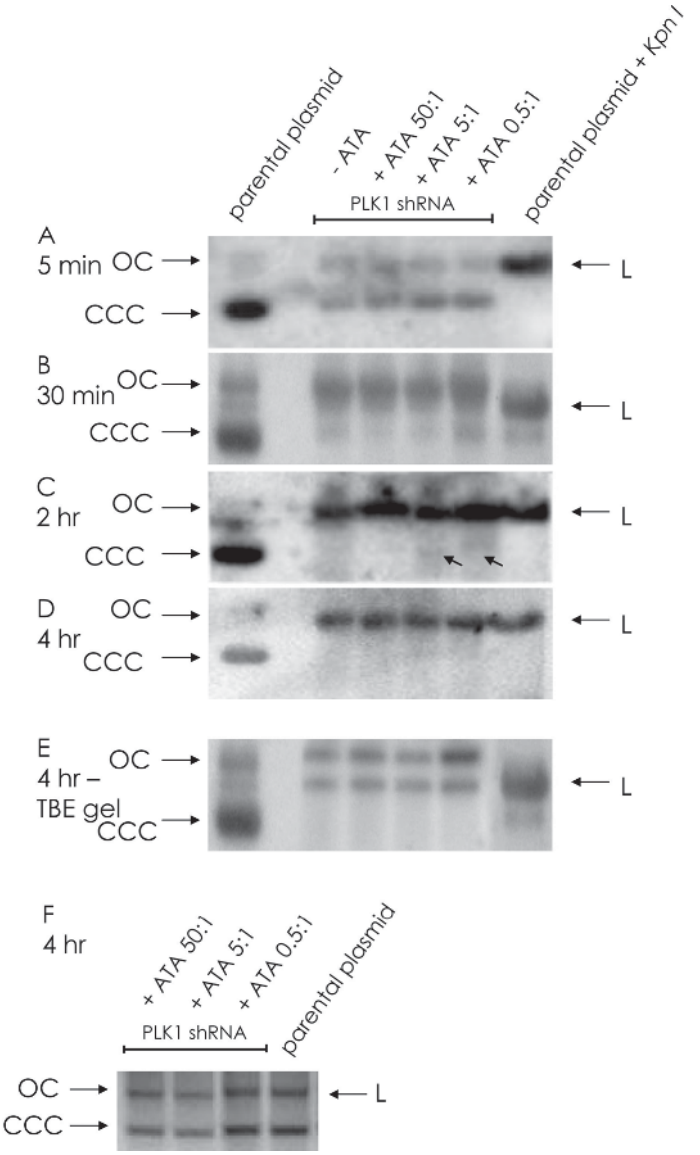


Figure 13.3 Nuclease inhibitor aurintricarboxylic acid (ATA) and the stability of plasmid DNA in murine blood. One milliliter of blood from a nude mouse was incubated at 37 °C with plasmids and ATA at the indicated weight ratios for 5 minutes (A), 30 minutes (B), 2 hours (C), or 4 hours (D); total DNA was isolated from the reaction mixture, separated by electrophoresis, and transferred to nylon membranes. The integrity of the plasmid DNA was then examined by Southern blot analysis. Control linearized (KpnI) and circular plasmids were also subjected to electrophoresis. Prolonged stability of supercoiled plasmid DNA is indicated by arrows (in panel C, DNA/ATA ratio = 5 : 1 and DNA/ATA = 0.5 : 1). (E) DNA was separated on 1% TBE gels after 4 hours of incubation with ATA at the indicated ratios for improved separation of linear and nicked circle forms; DNA was then examined by Southern blot analysis as described above. (F) Human blood was incubated at 37 °C with plasmids and ATA for 4 hours. The stability of plasmids was analyzed by ethidium bromide staining of the gels. L = linear DNA. OC = circular DNA. CCC = supercoiled DNA.

murine blood, stability was higher for ATA-treated supercoiled DNA than for untreated supercoiled DNA (Figure 13.3). Supercoiled DNA (CCC) was still visible after two hours at a ratio of DNA to ATA of 0.5 : 1, but it was at the limit of detection with lower ATA concentrations, such as a ratio of 5 : 1 (Figure 13.3 C, indicated by arrows). Southern blot analysis revealed that the corresponding degradation products (circular [OC] and linear [L] DNA) were detectable in murine blood for more than four hours (Figure 13.3 D). After a four-hour incubation and electrophoresis on TBE gels, the signal of circular DNA treated with the highest concentration of ATA was five times stronger than the signal of untreated circular DNA, indicating that ATA apparently protects plasmid DNA, especially circular DNA, in mammalian blood (Figure 13.3 E).

When higher concentrations of ATA were added to plasmid DNA before incubation with mouse blood, the degradation of plasmid DNA decreased in all samples in a concentration-dependent manner. Addition of ATA also protected the integrity of U6 promoter-containing vectors in human blood. After a four-hour incubation at 37 °C, the signal intensity of the supercoiled form [CCC] had increased from 75% at a ratio of DNA to ATA of 50 : 1 to 92% at a ratio of 0.5 : 1 relative to the signal intensity of a defined quantity of supercoiled plasmid (Figure 13.3 F). Thus, ATA protects plasmid DNA from degradation by nucleases in mammalian blood.

13.2.3 Antitumor Activity of PLK1 shRNA in vivo

To evaluate whether PLK1 shRNA from ATA-treated plasmids would inhibit PLK1 gene expression *in vivo* better than PLK1 shRNA from untreated plasmids, nude mice carrying subcutaneously implanted tumor xenografts (HeLa S3 and A549 cells) of 50–100 mm^3 were injected with untreated plasmids or with ATA-treated plasmids. Plasmids (PLK1 shRNA, control scrambled PLK1S shRNA, or parental control plasmids, each at 0.33–0.4 $mg \cdot kg^{-1}$ of body weight) were administered in 0.5 mL of PBS with or without ATA treatment to mice by bolus intravenous injection via the tail vein three times a week for 26 days. Administration of PLK1 shRNA plasmids statistically significantly reduced the growth of HeLa S3 tumors in mice in relation to treatment with control scrambled PLK1S shRNA plasmids or parental plasmids (Figure 13.4). When PLK1 shRNA plasmids were mixed with ATA at a ratio of 5 : 1 and the mixture was then administered to tumor-bearing mice, ATA-treated plasmids inhibited tumor growth more efficiently than untreated PLK1 shRNA plasmids. For the treatment period ending 42 days after transplantation, PLK1 shRNA expression from ATA-treated plasmids reduced tumor volume to 18% ($P = 0.03$) of tumor volume from mice injected with the ATA-treated scrambled control vector PLK1S shRNA. In contrast, PLK1 shRNA expression from untreated plasmids reduced tumor growth to only 45% ($P = 0.1$) of tumor volume from mice injected with the ATA-treated scrambled control plasmid. Thus, ATA treatment increased the inhibitory effect of PLK1 shRNA in the tumor xenografts. In addition, tumor growth (HeLa S3) did not resume during the first four weeks after treatment with ATA-treated PLK1 shRNA plasmids had ended. Four weeks after the end of

treatment, tumor volume in the group receiving ATA-treated PLK1 shRNA plasmids was reduced to 2.6% (P = 0.005) of that in the group receiving ATA-treated control scrambled PLK1S shRNA plasmids. Tumor volume in the group receiving untreated PLK1 shRNA plasmids was reduced to 17% (P = 0.04) of tumor volume in the group receiving ATA-treated control scrambled PLK1S shRNA plasmids.

No reduction in the body weight of mice treated with PLK1 shRNA or PLK1S shRNA plasmids (each at 0.33–0.4 mg · kg^{-1}) could be observed with or without ATA treatment. In addition, no histopathologic signs of adverse events in the heart, lung, liver, kidney, intestine, brain, bone marrow, or lymphatic tissues could be detected after treatment with ATA-treated PLK1 shRNA or PLK1S shRNA plasmids. Specifically, no pericarditis, myocardial fibrosis, signs of muscular dysfunction, valvular abnormality, or conduction disturbance – which can be detected in the heart during radiotherapy or chemotherapy – were found. The lung parenchyma was normal, with no sign of pneumonitis, fibrosis, or inflammation being found. No sign of inflammatory or regressive changes such as fibrosis were visible in the liver, and no evidence for tubulopathy, glomerulonephritis, or degenerative alterations was found in the kidneys. In the intestines, no sign of elevated cell death in the crypt epithelium, breakdown of the mucosal barrier, mucositis, or prominent compensatory or proliferative reaction could be detected. The brains appeared normal (no signs of vasculopathy or necrosis), and no altered proliferation and no immature cells were found in the bone marrow and lymphatic tissue.

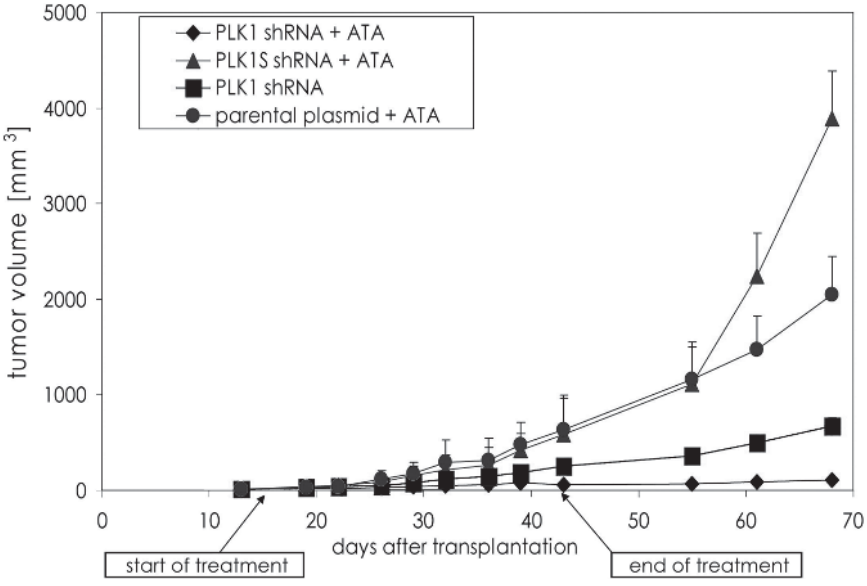


Figure 13.4 PLK1-specific short hairpin RNAs (shRNAs) driven by U6 promoters and the growth of HeLa S3 xenograft tumors in nude mice. HeLa S3 tumors were transplanted subcutaneously into the flanks of nude mice. Plasmids and ATA at a ratio of 5 : 1 were administered to tumor-bearing mice by bolus intravenous injection three times a week (Monday, Wednesday, and Friday) for 26 days.

The antitumor activity of PLK1 shRNA plasmids in nude mice implanted subcutaneously with A549 tumor xenografts was investigated by injection of A549 tumor-bearing mice with ATA-treated PLK1 shRNA plasmids at a weight ratio of 5 : 1. This resulted in inhibition of tumor growth to 9.8% (P = 0.002) in relation to tumor growth in mice receiving ATA-treated control PLK1S shRNA plasmids, in comparison with a growth reduction to 18.5% (P = 0.01) of tumor growth in mice receiving untreated control PLK1S shRNA plasmid. Some tumor growth could be observed in A549 tumor-bearing mice during the six weeks after injection with ATA-treated PLK1 shRNA plasmids had been terminated: the tumor volume in mice injected with ATA-treated PLK1 shRNA plasmids was 21% (P = 0.007) of that in mice injected with control scrambled PLK1S shRNA plasmids with ATA. Tumor volume in mice injected with untreated PLK1 shRNA plasmids reached 42% (P = 0.01) of that in mice injected with PLK1S shRNA plasmids with ATA. Thus, treatment of plasmids with ATA clearly enhanced the inhibitory effect of PLK1 shRNA in the HeLa S3 and A549 tumor xenografts without reducing the body weight of the mice.

13.2.4
Vector-Induced Decreased Expression of PLK1 and Antitumor Activity

To determine whether plasmid DNA was associated with the xenograft tumors, total DNA was isolated from HeLa S3 and A549 xenograft tumors and PCRs to detect plasmid DNA were performed. A 500-bp fragment was generated in PCR by use of plasmid (pBS/U6)-specific primers and tumor DNA from animals. Total tumor DNA from animals treated with the parental, PLK1 shRNA (with or without ATA) or PLK1S shRNA plasmids contained plasmid DNA, demonstrating that all plasmids could be found with xenograft tumor tissue *in vivo* (Figure 13.5).

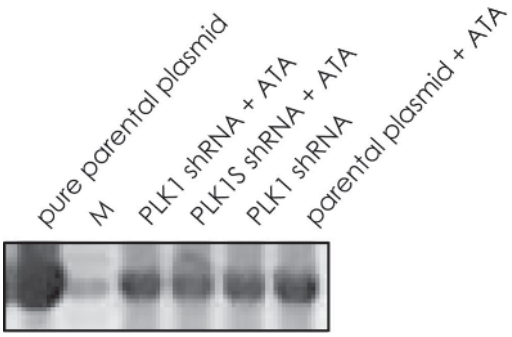


Figure 13.5 Analysis of tumors excised after termination of short hairpin RNA (shRNA) therapy. Detection of plasmids in the HeLa S3 tumors of mice from each of the four treatment groups. Primers against the parental plasmid were used as probes to detect all plasmids by polymerase chain reaction (PCR). Amplified products are shown after separation by electrophoresis. M = the DNA ladder.

To evaluate the effect of PLK1 shRNA on PLK1 mRNA expression in tumor cells, Northern blot analysis was used to measure PLK1 mRNA levels in total RNA that had been isolated from HeLa S3 xenograft tumors after a 26-day treatment with the parental, PLK1 shRNA, or scrambled PLK1S shRNA plasmids. Tumors of mice treated with PLK1 shRNA had lower levels of PLK1 mRNA than tumors of mice treated with the parental or scrambled plasmids (Figure 13.6 A). PLK1 mRNA expression was lower in tumors of mice injected with ATA-treated PLK1 shRNA plasmids (25%, $P = 0.007$) than in mice injected with untreated PLK1 shRNA plasmids (28%, $P = 0.02$), both relative to PLK1 mRNA expression in tumors of mice injected with ATA-treated scrambled control PLK1S shRNA plasmids. Results with mice carrying A549 tumors were similar to results with HeLa S3 tumors. PLK1 mRNA expression was lower in mice with A549 tumors injected with ATA-treated PLK1 shRNA plasmids (30%, $P = 0.02$) than in such mice injected with untreated PLK1 shRNA plasmids (60%, $P = 0.04$), both relative to PLK1 mRNA expression in mice treated with ATA-treated scrambled control PLK1 shRNA plasmids. To determine whether the reduced levels of PLK1 mRNA observed in HeLa S3 xenograft tumors treated with PLK1 shRNA reflect reduced PLK1 protein levels, Western blot analysis was used (Figure 13.6 B). Injection of mice with ATA-treated PLK1 shRNA plasmids statistically significantly reduced the level of PLK1 protein in HeLa S3 tumors to 15% ($P = 0.004$) of that detected in mice injected with ATA-treated control scrambled PLK1S shRNA plasmids, and injection of untreated PLK1 shRNA plasmids reduced the level of PLK1 protein to 24% ($P = 0.007$) of that detected in mice injected with ATA-treated control scrambled PLK1S shRNA plasmids. As with PLK1 mRNA expression, results with mice carrying A549 tumors were similar to results with HeLa S3 tumors. Injection of mice with ATA-treated PLK1 shRNA plasmids statistically significantly reduced the level of PLK1 protein in A549 tumors to 29% ($P < 0.001$) relative to that in mice treated with ATA-treated scrambled control PLK1S shRNA plasmids.

To test the vector system *in vivo* further, immunohistochemistry was carried out to measure the level of PLK1 gene expression in xenograft tumors. The percentage of PLK1-positive tumor cells was 0% ($P < 0.001$) in mice injected with ATA-treated PLK1 shRNA plasmids and 22.5% ($P = 0.006$) in mice injected with untreated plasmids. In contrast, 39.2% of tumor cells in mice injected with ATA-treated scrambled control PLK1S shRNA plasmids were PLK1 positive, and 31.3% of the tumor cells in mice injected with ATA-treated parental plasmids were PLK1 positive. Cell proliferation in tumors from the various treatment groups was assessed immunohistochemically by use of Ki-67 antibodies. The percentage of Ki-67-positive cells in HeLa S3 xenograft tumors in mice injected with ATA-treated PLK1 shRNA plasmids was 0% ($P < 0.001$), whilst that in tumors in mice injected with untreated PLK1 shRNA plasmids was 27.3% ($P = 0.03$), that in tumors in mice injected with ATA-treated scrambled control PLK1S shRNA plasmids was 44.8%, and that in tumors with ATA-treated parental plasmids was 32.3%. Thus, immunostaining of tumors for Ki-67 and PLK1 indicated that the antineoplastic effects observed in tumors with ATA-treated PLK1 shRNA plasmids were associated with a marked inhibition of HeLa S3 tumor cell proliferation.

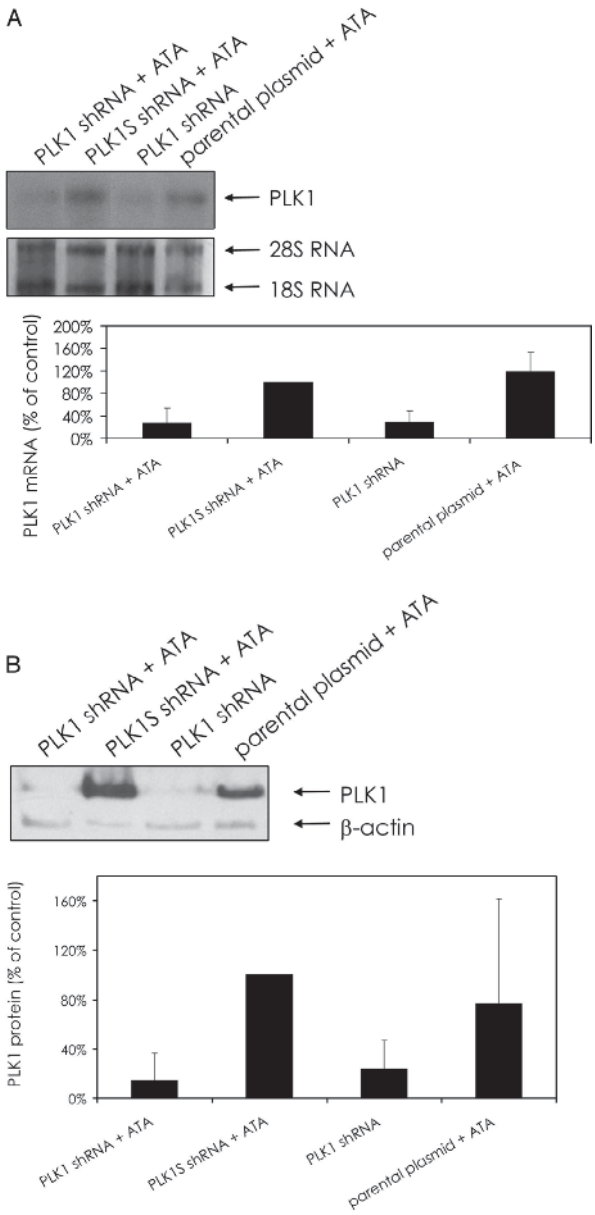


Figure 13.6 Polo-like kinase 1 (PLK1) protein expression in HeLa S3 xenograft tumors. Mice with xenograft tumors were treated with plasmids for 26 days. (A) Levels of PLK1 mRNA were determined in HeLa S3 tumors after 26 days of plasmid treatment. Tumors were excised 27 days after the beginning of treatment, total mRNA was isolated, and Northern blot analysis was performed. To control for variability of loading, gels were stained with ethidium bromide before blotting. (B) PLK1 protein expression in HeLa S3 tumors after 26 days of plasmid treatment. Tumors were excised 27 days after the beginning of treatment, total protein was isolated, and proteins were separated by electrophoresis, transferred to membranes, and examined by Western blot analysis with anti-PLK1 antibodies. To control for variability of loading, membranes were reexamined with antibodies against β-actin.

13.3 Conclusion and Future Directions

Although the feasibility and potential of siRNA in cancer therapy have not yet been demonstrated, siRNAs that have been chemically synthesized or inserted into plasmids have been shown to inhibit expression of transgenes, such as the gene for luciferase or the gene for green fluorescent protein, in adult mice (Brummelkamp et al., 2002; Lewis et al., 2002; McCaffrey et al., 2002; Xia et al., 2002). Current experimental evidence that siRNAs can inhibit endogenous genes is limited to genes expressed in murine liver, the *in vivo* silencing effect of siRNA directed against the Fas receptor gene having been tested for its potential to protect mice from liver failure and fibrosis in models of autoimmune hepatitis (Song et al., 2003). After administration of Fas-specific antibodies that induce fulminant hepatitis, all untreated control mice died within three days, whereas 85% of mice pretreated with Fas siRNAs survived, suggesting that RNA interference can prevent disease in an animal model of autoimmune hepatitis. RNA interference was also used to inhibit production of hepatitis B virus replicative intermediates in cell culture and in immunocompetent and immunodeficient mice transfected with a hepatitis B virus plasmid (McCaffrey et al., 2003). In another study, tail vein injection of adenovirus particles expressing murine-specific siRNAs against β-glucuronidase reduced the activity of β-glucuronidase in adult mice (Xia et al., 2002). Systemically administered adenovirus vectors can provoke immune responses, however, so their effectiveness for peripheral gene transfer is limited (Vorburger et al., 2002). The consequences of inappropriate vector integration must also be considered: despite the low integration efficiency, reports of viral mutagenesis in mice and in two human subjects have raised concern about the potential for recombinant adeno-associated virus-mediated genome integration (Li et al., 2002; Marshall, 2002, 2003).

In contrast, *in vivo* gene transfer of naked DNA is reproducible, simple, and safe, but degradation of the naked DNA by nucleases can be a problem. After numerous attempts in our laboratory, intravenous or intratumor injection of synthetic siRNA targeted to PLK1 failed to inhibit tumor growth in xenograft models (data not shown), probably because of the short halflives of PLK1-specific siRNAs. The PLK1-specific siRNA and its scrambled counterpart were completely degraded within 15 minutes of incubation in mammalian serum (data not shown). Stabilization of the siRNA by encapsulation in liposomes or coadministration with RNasin was not sufficient, because these protected siRNAs did not inhibit MCF-7 or SW-480 tumor growth in nude mice. Consequently, it was investigated whether shRNA vectors were more stable than siRNA in mouse serum and thus could suppress tumor growth in nude mice.

The studies described indicate that systemic administration of plasmid DNA carrying shRNA targeted to PLK1, even in the absence of nuclease inhibitors, reached the tumor and inhibited tumor growth. Treatment of plasmids with the nuclease inhibitor ATA and subsequent injection of these plasmids into mice increased the amount of plasmid DNA that reached the tumor. This observation is consistent with those demonstrating that DNA transfection of macaque, murine, and human respi-

ratory tissue can be enhanced by treating the DNA with ATA before administration (Glasspool-Malone et al., 2002). Information about the systemic application of ATA is limited, coming primarily from a study that investigated the effect of intravenous infusion of ATA on platelet aggregation in baboons (Alwayn et al., 2000). Although baboons receiving a daily dose of 24 mg of ATA per kg of body weight showed decreased platelet aggregation and increased coagulation time, baboons receiving 12 mg · kg^{-1} daily had normal blood parameters. No thrombotic disorders occurred in the experiments described above, in which mice received a much lower dose of ATA (80 μg · kg^{-1} of body weight) infused with plasmid DNA three times a week.

In conclusion, this was the first demonstration that U6 promoter-driven shRNAs targeted against PLK1 integrated into a bacterial plasmid suppress tumor growth in mice when administered intravenously with the nuclease inhibitor ATA. The combination of shRNA-mediated gene silencing with effective *in vivo* gene delivery strategies appears to generate a longlasting silencing signal.

A recent report documents the use of chemically modified siRNA for down-regulation of apoB mRNA and protein expression in liver and jejunum, of plasma levels of apoB protein and of total cholesterol after intravenous injection in mice (Soutschek et al., 2004). Chemically stabilized siRNAs applied in this trial include a partial phosphorothioat backbone and 2′-*O*-methyl sugar modifications on the sense and antisense strands as well as conjugation of cholesterol to the 3′-end of the sense strand of a siRNA molecule through a pyrrolidine linker. These modifications improved the pharmacological properties of siRNAs (enhanced resistance towards degradation by exo- and endonucleases in serum and tissue homogenates, improved cell penetration ability) both *in vitro* and *in vivo*. Mice received siRNAs at doses of 50 mg · kg^{-1} in 0.2 mL per injection. Mice treated with chol-apoB-1-siRNA showed reductions between 36 and 57% in apoB mRNA levels. These data suggest that further optimization is required to achieve improved *in vivo* potency of chol-siRNAs at clinically acceptable doses and dose regimens.

Thus, only our findings based on the use of plasmids for the expression of hairpin RNA currently hold promise for the development of a new class of therapeutics harnessing the RNAi mechanism.

References

Alwayn, I. P., Appel, J. Z., Goepfert, C., Buhler, L., Cooper, D. K., Robson, S. C., *Xenotransplantation* **2000**, *7*, 247–257.

Blumenthal, T., Landers, T. A., *Biochem. Biophys. Res. Commun.* **1973**, *55*, 680–688.

Brummelkamp, T., Bernards, R., Agami, R., *Cancer Cell* **2002**, *2*, 243.

Chong, S. S., Hu, P., Hernandez, N., *J. Biol. Chem.* **2001**, *276*, 20727–20734.

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., Tuschl, T., *Nature* **2001**, *411*, 494–498.

Glasspool-Malone, J., Steenland, P. R., McDonald, R. J., Sanchez, R. A., Watts, T. L., Zabner, J., Malone, R. W., *J. Gene Med.* **2002**, *4*, 323–332.

Glover, D. M., Hagan, I. M., Tavares, A. A., *Genes Dev.* **1998**, *12*, 3777–3787.
Hallick, R. B., Chelm, B. K., Gray, P. W., Orozco, E. M., Jr., *Nucleic Acids Res.* **1977**, *4*, 3055–3064.
Hannon, G. J., *Nature* **2002**, *418*, 244–251.
Hannon, G. J., Chubb, A., Maroney, P. A., Hannon, G., Altman, S., Nilsen, T. W., *J. Biol. Chem.* **1991**, *266*, 22796–22799.
Lee, N. S., Dohjima, T., Bauer, G., Li, H., Li, M. J., Ehsani, A., Salvaterra, P., Rossi, J., *Nat. Biotechnol.* **2002**, *20*, 500–505.
Lewis, D. L., Hagstrom, J. E., Loomis, A. G., Wolff, J. A., Herweijer, H., *Nat. Genet.* **2002**, *32*, 107–108.
Li, Z., Dullmann, J., Schiedlmeier, B., Schmidt, M., von Kalle, C., Meyer, J., Forster, M., Stocking, C., Wahlers, A., Frank, O., Ostertag, W., Kuhlcke, K., Eckert, H. G., Fehse, B., Baum, C., *Science* **2002**, *296*, 497.
Lobo, S. M., Ifill, S., Hernandez, N., *Nucleic Acids Res.* **1990**, *18*, 2891–2899.
Marshall, E., *Science* **2002**, *298*, 34–35.
Marshall, E., *Science* **2003**, *299*, 320.
McCaffrey, A. P., Meuse, L., Pham, T. T., Conklin, D. S., Hannon, G. J., Kay, M. A., *Nature* **2002**, *418*, 38–39.
McCaffrey, A. P., Nakai, H., Pandey, K., Huang, Z., Salazar, F. H., Xu, H., Wieland, S. F., Marion, P. L., Kay, M. A., *Nat. Biotechnol.* **2003**, *21*, 639–644.
McManus, M. T. and Sharp, P. A., *Nat. Rev. Genet.* **2002**, *3*, 737–747.
Paul, C. P., Good, P. D., Winer, I., Engelke, D. R., *Nat. Biotechnol.* **2002**, *20*, 505–508.
Song, E., Lee, S. K., Wang, J., Ince, N., Ouyang, N., Min, J., Chen, J., Shankar, P., Lieberman, J., *Nat. Med.* **2003**, *9*, 347–351.
Soutschek, J., Akinc, A., Bramlage, B., Charisse, K., Constien, R., Donoghue, M., Elbashir, S., Geick, A., Hadwiger, P., Harborth, J., John, M., Kesavan, V., Lavine, G., Pandey, R. K., Racie, T., Rajeev, K. G., Rohl, I., Toudjarska, I., Wang, G., Wuschko, S., Bumcrot, D., Koteliansky, V., Limmer, S., Manoharan, M., Vornlocher, H. P., *Nature* **2004**, *432*, 173–178.
Spankuch-Schmitt, B., Bereiter-Hahn, J., Kaufmann, M., Strebhardt, K., *J. Natl. Cancer Inst.* **2002**, *94*, 1863–1877.
Strebhardt, K., PLK (polo-like kinase). In: Creighton, T. E. (Ed.), *Encyclopedia of Molecular Medicine*. New York, N. Y.: Wiley and Sons, Inc. **2001**, 2530–2532.
Sui, G., Soohoo, C., el Affar, B., Gay, F., Shi, Y., Forrester, W. C., Shi, Y., *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 5515–5520.
Tuschl, T., *Nat. Biotechnol.* **2002**, *20*, 446–448.
Vorburger, S. A., Hunt, K. K., *Oncologist* **2002**, *7*, 46–59.
Xia, H., Mao, Q., Eliason, S. L., Harper, S. Q., Martins, I. H., Orr, H. T., Paulson, H. L., Yang, L., Kotin, R. M., Davidson, B. L., *Nat. Med.* **2004**, *10*, 816–820.
Xia, H., Mao, Q., Paulson, H. L., Davidson, B. L., *Nat. Biotechnol.* **2002**, *20*, 1006–1010.

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