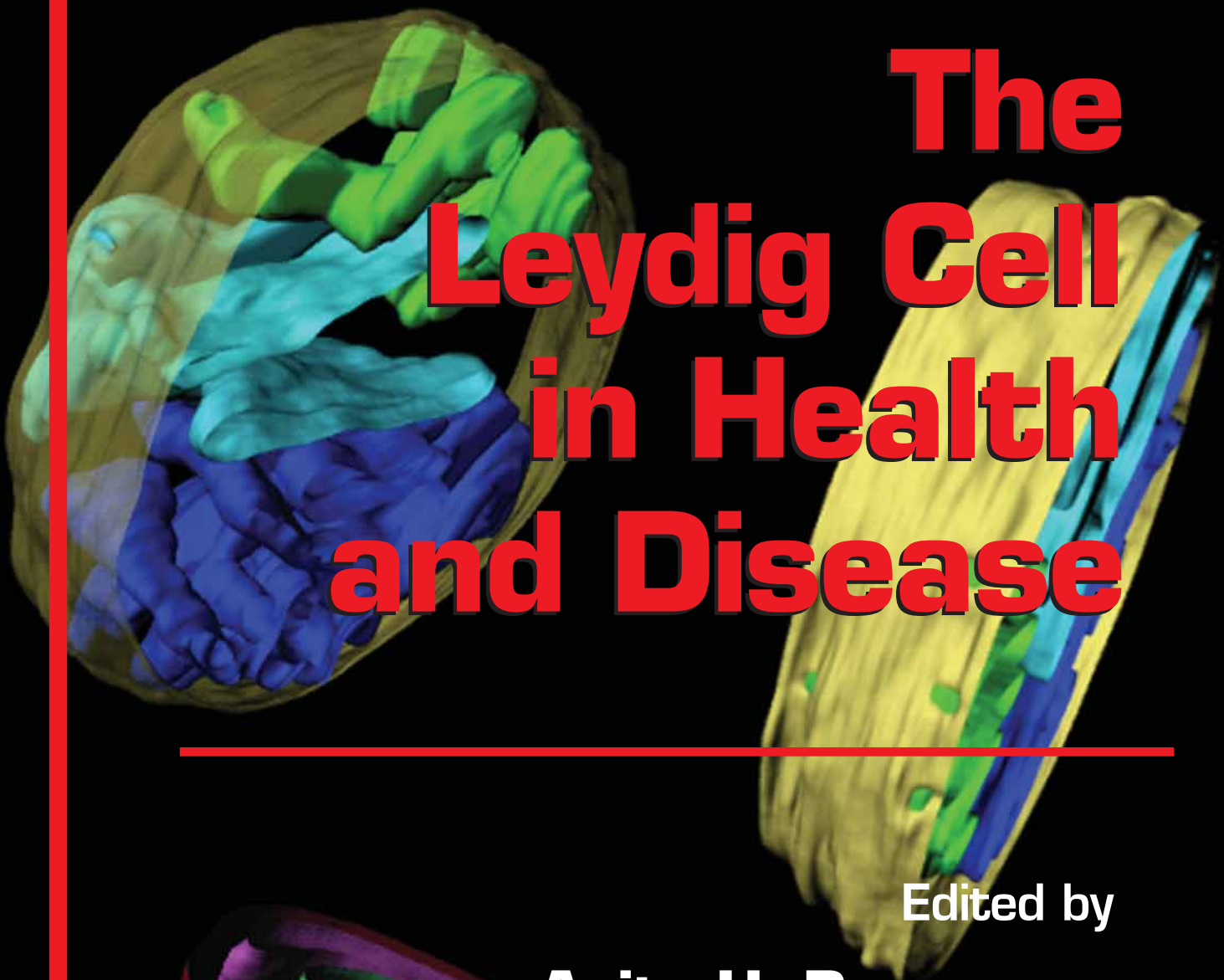
CONTEMPORARY ENDOCRINOLOGY™



The Leydig Cell in Health and Disease

Edited by

Anita H. Payne, PhD

Matthew P. Hardy, PhD

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The Leydig Cell in Health and Disease

CONTEMPORARY ENDOCRINOLOGY

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The Leydig Cell in Health and Disease

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PREFACE

Ten years ago, in association with Lonnie Russell, we published *The Leydig Cell* (Cache River Press, Vienna IL). It was the first volume of its kind, devoted entirely to review chapters covering basic and clinical aspects of Leydig cell structure and function. As editors, it became clear to us that such a book filled a gap in the literature on male reproduction. The testis produces both sperm and testosterone, and the two are linked because spermatogenesis is critically dependent on androgen action. However, the role of the Leydig cell in synthesizing the male hormone can be lost in the beautiful and complex series of cellular events leading to the formation of sperm. In addition, Leydig cells, which are far outnumbered by the germ cell population, constitute only 5 to 15% of the testicular volume (depending on the species). Despite their minority status in the testis, knowledge of the biology of Leydig cells is an essential prerequisite to understanding male fertility, sexual function, and reproductive health. And yet, there was no centralized repository for that knowledge. The first book, then, was an attempt to fill this unmet need.

Ten years have passed and science has advanced, but the question arises as to why it would be appropriate and advisable to publish a second volume, again focusing on this one cell type. We would argue that successes in the field have created a new gap. In each of the 30 chapters of *The Leydig Cell in Health and Disease*, we aim to provide closure while simultaneously pointing to new possibilities. We hope to make the case that the Leydig cell merits the reader's time and attention as much as it did in 1996. It would be difficult to highlight some of the developments that made the new book worthwhile without going beyond the scope of a preface and writing a 31st chapter. In more recent years the crystal structures of a gonadotropin receptor and several of the steroid synthesizing and metabolizing enzymes have been solved. In some cases, research groups working at biopharmaceutical companies achieved the structure solutions. Obviously, the potential for applying results related to androgen synthesis to drug development and therapy has not gone unnoticed in the private sector.

We know more about the process of cholesterol transport to the inner mitochondrial membrane and site of side-chain cleavage, the rate-limiting step in testosterone biosynthesis. It appears in 2006 to be like a Virginia reel dance in which the precursor, cholesterol, may arrive with one binding partner, the steroid acute regulatory protein, but finish with another, the peripheral benzodiazepine receptor, which has been clearly shown to form a pore in the mitochondrial membrane. How the cytosol to mitochondrial membrane transfer occurs remains an actively debated research topic.

We are also on the verge of a new male contraceptive after more than 30 years of studying the hormonal approach. Essentially, this method will involve using androgen in combination with another sex steroid to impose negative feedback on the hypothalamus and pituitary Leydig cell function will then be suppressed, lowering testicular testosterone levels below the threshold needed to sustain spermatogenesis. It will soon be known whether this is an effective product and one that will find a niche in the marketplace. Meanwhile, use and abuse of androgen for its effects as an anabolic steroid to increase muscle mass is a phenomenon among professional athletes and teenage boys. The fertility-suppressing effects of exogenous androgen are a potential, if unrecognized, concern within this group.

Whereas boys and younger men seek to boost androgen levels above normal, a potentially more legitimate trend exists for men that are aging. With aging, androgen levels decrease as a result of atrophic changes in Leydig cells. Administering testosterone can counteract the decline. Longitudinal studies, currently in progress, involving testosterone replacement in the aging male will become more acceptable if proven beneficial and safe.

It is now more apparent than it was in 1996 that exposure to environmental toxicants interferes with Leydig cell function. Some of the toxicants in this category, termed endocrine disruptors, are now known to antagonize testosterone synthesis (phthalate esters, for example). It is of vital importance to put all of the above developments into a rigorous scientific perspective, and

the present volume has additional emphasis on clinical applications. We hope to have succeeded in this endeavor. Special thanks are due to Meghan Howard and Jean Schweis at the Population Council for their expertise in the management of the manuscripts as they were received from the authors, and to Richard Lansing of Humana Press for his expert advice during all phases of production. We also are grateful to Michael Conn for leading us to Humana Press and for including *The Leydig Cell in Health and Disease* in the Contemporary Endocrinology™ series. Finally, we thank the authors and commend their work to our readers.

Anita H. Payne
Matthew P. Hardy

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Color Plate 2. *Fig. 3, Chapter 1:* Plate from Reinke's 1896 article describing crystalloids in human Leydig cells. (*See* full caption on p. 10.)

Color Plate 3. *Fig. 4, Chapter 1:* First plate from Bouin and Ancel's 1903 article showing testes of piglets of suckling age. (*See* full caption on p. 15.)

Color Plate 4. *Fig. 1, Chapter 2:* FLCs in rat testis. (*See* full caption on p. 34.)

Color Plate 5. *Fig. 6, Chapter 2:* Bar for **(A–D)**: 10 μm. **(A,B)**: Immunohistochemical reaction of procollagen. (*See* full caption on p. 40.)

Color Plate 6. *Fig. 1, Chapter 3:* Origin, specification, and differentiation of FLC. (*See* full caption on p. 48.)

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I Historical

1 A History of Leydig Cell Research

A. Kent Christensen, PhD

SUMMARY

Franz Leydig first described the testicular cells in 1850 that now bear his name. For the next 50 yr after their discovery, Leydig cells were the subject of further studies by light microscopy, and diverse speculations were offered about their possible function. In 1903, Pol Bouin and Paul Ancel provided the first substantial evidence that Leydig cells constituted an endocrine gland controlling male secondary sexual characteristics. Their evidence seemed compelling at the time, but was necessarily circumstantial, because there was no direct proof that Leydig cells produced a male hormone. Over subsequent decades, workers found additional evidence that these cells had an endocrine function, but there were also other findings that cast doubt on the hypothesis, and increasing skepticism developed about the earlier evidence. By the late 1920s, many influential reproductive biologists suspected that the seminiferous tubules were the actual source of male hormone. During the 1930s, the male hormone was shown to be testosterone, its endocrine actions were studied extensively, and the role of the pituitary in regulating testicular function was demonstrated. From the 1930s through the 1950s, Leydig cells came back into favor as endocrine cells, although some uncertainty persisted and there was still no direct evidence that Leydig cells produced androgen. Finally, direct evidence came from histochemistry in 1958 and from biochemistry in 1965.

Key Words: Ancel; androgen; Bouin; endocrine; histology; history; Leydig; male; testis; testosterone.

INTRODUCTION

In 1850, Franz Leydig described cells in the mammalian testis *(1)* that were later shown to be the source of testicular hormones that control spermatogenesis, the male reproductive tract, and male secondary sexual characteristics. These Leydig cells, sometimes called interstitial cells *(2)*, occur in clusters between the seminiferous tubules. Leydig's investigations were part of the great elaboration of histology that took place in the years after Schleiden *(3)* and Schwann *(4)* proposed the cell theory in 1838–1839. An earlier technical advance in microscope lenses—the development of achromatic objective lenses in the 1820s—had opened the way for both the cell theory and histology because microscopists could now actually observe cells relatively well *(5–7)*. However, specimen preparation was still rudimentary, and tissues were usually observed in manually cut slices, or in teased or macerated preparations *(8)*. But the foundations of histology were laid during this era, and Franz Leydig was one of the pioneers in these developments.

FRANZ LEYDIG (1821–1908)

Franz Leydig (refs. *9–15*; Fig. 1), was born on May 21, 1821, in Rothenburg ob der Tauber, a charming German town surrounded by a medieval wall. His father was a clerk in a municipal salt shop *(Salzamtsdiener)*, and adjacent to it owned a piece of land that included a small garden, highly-regarded locally for its beauty. The young Franz was an ardent naturalist who loved nothing better than to search for worms, beetles, and other wildlife in that family garden and field. He knew the scientific names of most of the animals and plants he encountered. He also enjoyed observing tiny objects with a small microscope that had been purchased for him when he was about 12 yr old; it had been made in Nürnberg, with lenses held in wooden mounts and with a cardboard microscope tube.

He went to elementary school *(Volksschule)* and then to Latin school *(Lateinschule)*. In Latin school he was the only student in the class and, as a consequence,

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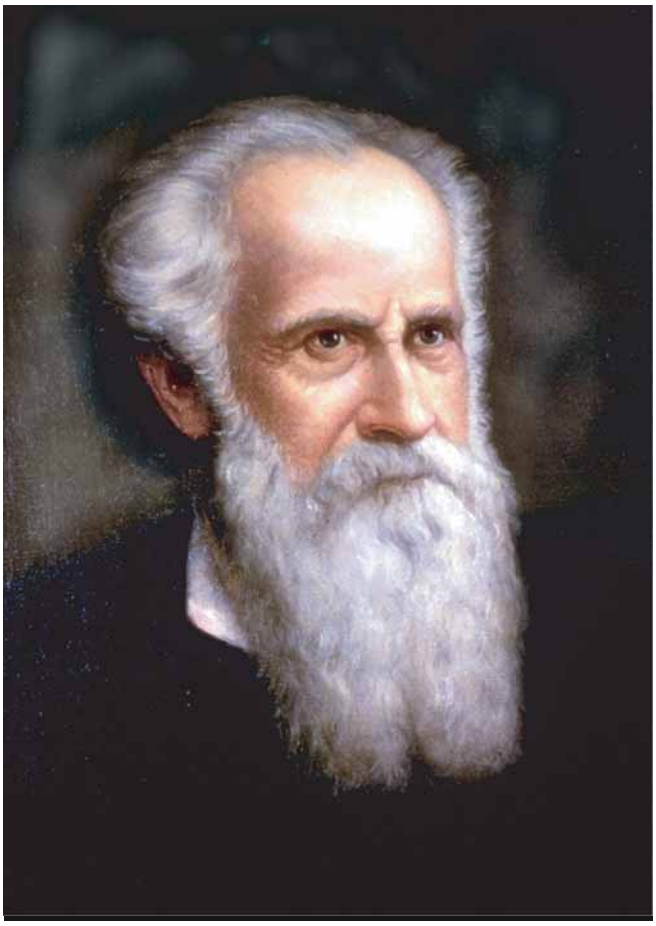


Fig. 1. Portrait of Franz Leydig in his later years, from a painting that has hung in the library of the Institute of Anatomy at the University of Bonn since the days when Leydig was professor there (1875–1887). (Please *see* color version of this figure on color insert following p. 180.)

much of his learning from Rothenburg professor Benzen, an inspiring teacher and historical scholar, occurred during daily afternoon walks out in the countryside. This training and his natural ability allowed Franz to enter the Bamberg Gymnasium at the age of 15, and to finish in 3 yr.

In 1840, Franz Leydig entered the University of Munich as a student in philosophy, then a broad subject including mostly sciences. He intended to emphasize zoology in his studies. However, the zoology lessons he received did not meet his expectations, amounting, as he later quipped, mostly to the study of bird bones on his Sunday dinner plate, and fish bones at his Friday meal. After 2 yr, it was clear to Leydig, at the age of 20, that this was not what he wanted to do with his life, and so he gave up his scholarship and in the summer of 1842 began medical studies at the University of Würzburg.

At Würzburg, he studied under exceptional teachers, that included human anatomy from professor Martin Münz, botany from Friedrich Schenk, and especially a variety of subjects from the highly-regarded clinician Franz von Rinecker (1811–1883). By 1846, to help defray expenses, he had become an assistant in the Physiology Institute, and was also helping out in various anatomy courses. Research in microscopic anatomy was not very well developed at Würzburg at that time. In order to build in that field, and allow research and training, von Rinecker in 1846 decided to establish the beginnings of a "Microscopy Institute," and asked Franz Leydig to play a major role in setting it up. A small room alongside the gross anatomy area was designated for that purpose. Leydig was able, with advice and help from von Rinecker, to locate a small Oberhäuser microscope with which to outfit the room, and an old wooden incubator (heated by a small oil lamp) that had previously been used for embryology teaching. The microscope had been manufactured by the prominent firm of Georg Oberhäuser, a German living in Paris, whose company at about that time was playing an important role in bringing light microscopes into the general design they have today, by modifying tube length and the base *(6)*. The availability of this room and equipment undoubtedly aided Leydig's development as a histology researcher, and gave him the means to conduct investigations that would lead to many findings, including the discovery of Leydig cells.

Leydig received his medical degree (Doctor of Medicine) in 1847, after defending three theses and presenting a dissertation entitled "Yolk cleavage, its origin in the animal world and its significance" *(Die Dotterfurchung nach ihrem Vorkommen in der Tierwelt und nach ihrer Bedeutung)*. This work was published by Leydig in 1848 *(16)* and has been said *(6)* to contain the first clear description of nuclear division. By this time, Leydig had a position as an assistant in the small Microscopy Institute that he had set up under von Rinecker's direction.

In the fall of 1847, the medical faculty at Würzburg was greatly strengthened by the arrival of a new professor, Rudolph Albert Kölliker (1817–1905) *(17,18)*, an internationally renowned histologist from the University of Zurich, who would be in charge of physiology, anatomy, comparative anatomy, histology, and embryology in the medical faculty at the University of Würzburg. Kölliker was originally hired as the professor of physiology and of comparative anatomy, but when professor Münz died in 1849, Kölliker also replaced him as professor of anatomy. From the time Kölliker arrived at Würzburg, he placed high priority on building up the Microscopy Institute, with Leydig as one of his principal aids in that endeavor. Kölliker

later wrote (in translation): "My friend Rinecker had already set up a Microscopy Institute, with his assistant Leydig, as the first such institute anywhere (not only in Germany). I and Leydig, that meritorious researcher who unfortunately is not with us today *[welchen hochverdienten Forscher ich leider unter den heute Anwesenden vermisse]*, further developed this institute such that it could serve as a model for all subsequent organizations of that kind" (ref. *17*, p. 39). Kölliker also organized a microscopy course, "strongly aided by Leydig" *(17)*, which was a research course dealing with the use of microscopes, preparation methods for various organs, and relevant histology. This was the first course of its kind offered in Germany or perhaps anywhere else *(17)*. After its beginning in the summer of 1848, the course thereafter was usually held in the winter semester from 6 to 8 PM, twice a week. In Kölliker's autobiography (ref. *17*, p. 33), he spoke of Franz Leydig as one of the three particularly able young men whose help proved highly valuable in Kölliker's efforts during his first decade at Würzburg, to establish a world-famous research and academic center.

Leydig became a prosector in anatomy at Würzburg in 1848. He then applied for a faculty position as lecturer *(privatdozent)*, which had to be earned by a grueling procedure *(habilitation)* that included submitted writings, a competitive examination, and a lecture presentation. The topic he chose for his lecture, delivered in April 1849, was "On the structural relationships of Cowper's gland and prostate in various mammals" *(Ueber die Strukturverhältnisse der Cowperschen und Vorsteherdrüse bei den verschiedenen Säugetieren)*, showing histological results from his studies on the male reproductive system. He was appointed lecturer in June 1849. The rank of lecturer *(privatdozent)* would be roughly comparable to an untenured assistant/associate professor in an American medical school today.

Also in 1849, Rudolph Virchow came to the University of Würzburg from the Charité Hospital in Berlin as the new professor of pathology, further strengthening the medical faculty and enhancing the scientific environment in which Leydig's career was developing.

It was about this time that Leydig submitted a manuscript, describing his comparative histological studies on the male reproductive system in various mammals, for publication in the *Zeitschrift für wissenschaftliche Zoologie,* a journal edited by von Siebold and Kölliker. The article included the first description of the testicular cells that would come to bear Leydig's name. It appeared in early 1850, *(1)* and will be described in more detail later.

Late in 1850, Leydig received a royal Bavarian grant of 500 florins for a half year trip down the Italian Mediterranean coast as far as Naples, as well as to the island of Sardinia, to study comparative histology and embryology in terrestrial and marine animals. The stipend was further supplemented by funds from the Würzburg medical faculty and senate. The trip was an extremely valuable experience for Leydig, and broadened his research outlook beyond mammals to other vertebrates and invertebrates.

Leydig aspired to a permanent faculty position at the University of Würzburg, but in the German academic system there was only one regular professor *(ordentlicher Professor)* in each institute, and Kölliker held that position for the Anatomy and Physiology Institutes at Würzburg. However, a lecturer could achieve permanent faculty status as a nonregular or "extraordinary" professor *(ausserordentlicher Professor)*. Leydig applied to the Bavarian State Ministry for this rank in March 1853 and, after lengthy negotiations to demonstrate his worthiness, and the need at Würzburg, he was finally awarded an extraordinary professorship in May 1855, at twice the salary that had been requested.

In August of 1855, Franz Leydig married Katharina Jaeger, daughter of a former Würzburg surgeon who had become professor of surgery at the University of Erlangen before his death in 1838. Franz and Katharina had a happy life together, but did not have any children. Leydig's histology textbook *(19)* appeared in 1857. It was entitled *Lehrbuch der Histologie des Menschen und der Thiere* (Textbook of Human and Animal Histology). It was a treatise on comparative histology, describing the microscopic anatomy of the body in man and a variety of vertebrates and invertebrates. Leydig was indeed the father of comparative histology. The earlier histology textbooks of Joseph von Gerlach in 1848 *(20)* and of Albert Kölliker in 1850/1854 *(21)* and in 1852 *(22)* had dealt primarily with human histology.

In the summer of 1857, Franz Leydig left Würzburg to become the professor of zoology and comparative anatomy at the University of Tübingen, replacing Wilhelm Rapp, who had died the previous year. Leydig had been recommended for the position by the famous Johannes Müller of Berlin, who was acting on the advice of one of his former students, Hugo von Mohl, professor of botany at Tübingen. Leydig taught and continued his research in comparative histology for 18 yr at Tübingen. In 1869, he was offered the zoology professorship back at Würzburg, but declined the offer.

In 1875, Leydig accepted a professorship in the Anatomy Institute at the University of Bonn, a position that had become vacant because of the death of Max

J. S. Schultze in 1874. It was decided to replace Schultze with two professors, thus dividing the Institute of Anatomy into two divisions. Leydig became the professor of comparative anatomy, histology, and embryology, and Adolph von la Valette St. George became the professor of normal human anatomy. After the death of zoology's former chair in 1882, and the departure of his replacement, Richard Hertwig 3 yr later, in 1885, Leydig became the professor of zoology, in the philosophy faculty.

At Bonn, Leydig continued his research, and taught microscopic anatomy, and other subjects to medical students. He was remembered as an outstanding teacher. It is said that his students revered him *(12)*, and that at later reunions of medical alumni from the University of Bonn *(13)*, the older alumni remembered Dr. Leydig as one of their most inspiring teachers. Among other things, his students remembered the remarkable blackboard drawings he made, often with colored chalk, to illustrate important points. Figure 1 is a copy of an oil painting of the older Leydig that has hung in the library of the Anatomy Institute at the University of Bonn since the days when Leydig was professor there. A similar engraving had been published elsewhere *(10)*.

Leydig retired from active academic life in 1887, at the age of 66. For several years, he and his wife spent their winters in Würzburg, her hometown, where he could continue his research. The townpeople of Würzburg venerated the aging professor. In the words of someone who grew up in Würzburg during that era (in translation): "I can still hear the admonishing words of my Mother, as she was leading us children along Theater Street in Würzburg: 'Children, now you must take off your caps. Here comes the old Leydig'" *(4)*. During many of the summers Leydig and his wife lived in his hometown, Rothenburg ob der Tauber, about 50 km south of Würzburg. In 1896, after a serious bout of influenza had sapped Leydig's strength, they moved permanently to Rothenburg. Franz Leydig died there on April 11, 1908, at 87 yr of age.

During his research career, Leydig produced some 200 publications (for a complete list, *see* ref. *15*), dealing with a broad range of topics covering most of the major groups of animals, from single-celled organisms to mammals. Usually, his articles were illustrated with drawings he did himself. Because of his wide-ranging interests, Leydig was the first to describe many structures in a variety of animals, and some of these new structures were subsequently named after him. Another "Leydig cell," that has nothing to do with testicular Leydig cells except having been discovered by the same man, is the dermal Leydig cell of the amphibian epidermis. These large, striking, granule-containing cells were first described by Leydig in the larval skin of the salamander *Salamandra maculosa* in 1853 (ref. *23*, pp. 107–108), and were later discussed more broadly in 1876 article (ref. *24*, pp. 144–146). Although Leydig called them "mucous cells" *(Schleimzellen),* the function of these dermal Leydig cells is still uncertain.

THE ORIGINAL DESCRIPTION OF TESTICULAR LEYDIG CELLS (1850)

Franz Leydig's 1850 article *(1)*, published while he was a lecturer at Würzburg, was a comparative study of male reproductive histology in various mammals. The article dealt mainly with the male tract and its accessory glands, thus focusing on seminal vesicle, prostate, epididymis, ductus deferens, Cowper's gland, uterus masculinus, and also anal glands. In addition, he made comments on some aspects of the testis, although the histology of sperm and spermatogenesis in the seminiferous tubules had already received a great deal of attention over the preceding two decades, including pioneering work by Kölliker *(25,26)*. Leydig's remarks on the testis referred to its vasculature, pigmentation, appearance of the seminiferous tubules, and also to prominent clusters of what appeared to be cells, overlooked by previous researchers, that he found consistently between the seminiferous tubules. These of course were the cells that have come to be called "Leydig cells," the subject of this volume.

The article is organized on the basis of major mammalian groups, dealing successively with (using common names): nonhuman primates, bats, insectivores, carnivores, marsupials, rodents (and rabbits), pigs, horses, artiodactyls, and a dolphin. Occasional references to Leydig cells are, therefore scattered through the 53 pages of text. Specific descriptions of the cell clusters are given for bat, mole, cat, rat, mouse, rabbit, pig, and horse. Near the end of the article is a 12-page summary of findings for the various organs, including a page on the testis, with a 13-line summary about Leydig cells (ref. *1*, pp. 47–48): "From the comparative histology of the testis it is clear that, in addition to seminiferous tubules, blood vessels, and nerves, one finds an additional constant component in the mammalian testis, namely a cell-like mass that when present in smaller amount follows the course of blood vessels between the seminiferous tubules, but when more developed, becomes a mass in which the seminiferous tubules are embedded. Its main constituents are small

granules of fatty appearance, which are unaltered by acetic acid and sodium hydroxide treatment, are colorless, or yellowish, and encompass clear, bubble-like nuclei. Its semifluid ground substance may condense into a cell membrane, and at least in some mammals the entire granular mass is surrounded by a sharp outline. Also, at times the entire structural aggregate is of such an appearance that one can speak of it as a complete cell."

Of the 43 figures, arranged in four plates, only two figures by Leydig, Figs. 6 and 7, show Leydig cells. These two figures are reproduced here, considerably enlarged, as Fig. 2. The legend for Fig. 2 includes an English translation of Leydig's legends for his Figs. 6 and 7. If you want to visualize a facsimile of the original German text of the testis summary (ref. *1*, pp. 47–48), as well as a complete copy of his Plate 1 (Figs. 1–13) and its German legends, consult Setchell's *Male Reproduction* (ref. *26*, pp. 230–235). The article by Ober and Sciagura *(10)* includes a facsimile of the title and first paragraph of Leydig 1850, as well as an enlargement of Figs. 6 and 7.

The general features of Leydig's description agree with what we know today. However, an understanding of the text in Leydig's article requires some acquaintance with the procedures of tissue preparation for microscopy that were current in the late 1840s, when Leydig made these observations. At that time, the available techniques did not allow a very clear view of tissues. The method of tissue preparation that we take for granted today—fixation, dehydration, embedding, sectioning, staining, and viewing with an oil immersion lens—would not be developed until many years after Leydig's observations were made. Instead, common procedures of that era *(8)* included teasing living tissues apart with fine needles, or cutting manual slices as thin as possible of living tissue, or of organs that had been hardened in alcohol (usually a poor histological fixative), or other substances such as chromium trioxide. Another approach was to mascerate pieces of tissue by leaving them in various solutions long enough to break down some of the connective tissue, allowing components to be teased apart. Generally, contrast was poor, but could be improved somewhat (at the expense of resolution) by lowering the position of the microscope's condenser (or closing a condenser diaphragm, if present), or by using one of the rather nonspecific stains available at the time, such as carmine, cochineal, saffron, madder, or indigo. The microscope's achromatic objective lens was used dry (no oil immersion yet). Although coverslips (of thin glass or mica) had been used before this time, it is not clear whether or not Leydig used them; if not, then irregular water contours on the surface of the specimen being viewed would have further reduced the quality of the image.

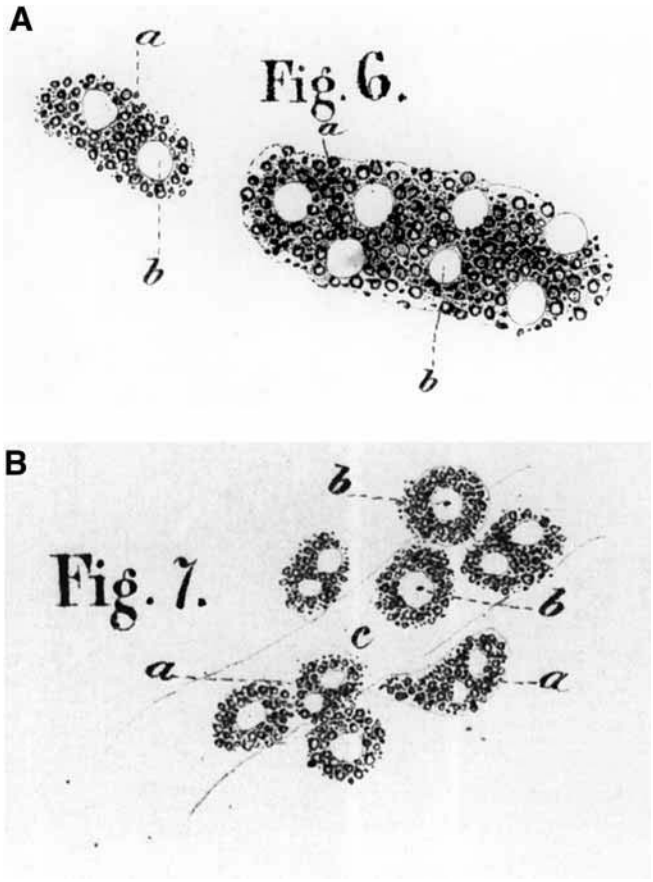


Fig. 2. Illustration of Leydig cells from the original Leydig 1850 article *(1)* in which the cells were first described. Leydig's Figs. 6 and 7 (from his first plate) are shown here, the only figures of Leydig cells in the article. The two figures have been enlarged here about 2.5 times their original size. The following are translations of the legends Leydig provided for his figures: "Figure 6. Masses found between the seminiferous tubules and also in the mediastinum in the cat testis. **(A)** Small fat granules, embedded in a soft ground substance. **(B)** the clear vesicular nuclei that are enclosed in the masses. Figure 7. The same masses seen in the testis of the bat, *Vesperugo pipistrellus*. **(A,B)** These have the same meaning as in Fig. 6. **(C)** A blood vessel on which the masses are usually situated."

In the preparations available to him, Leydig often found it difficult to make out intercellular boundaries between the Leydig cells, or to discern a sharp contour at the surface of cells or masses that would suggest a cell membrane. Most of the "fatty granules" he described filling the cytoplasm of Leydig cells were probably lipid droplets, abundant in the Leydig cells of many species, and which would have been visible as refractile structures in teased living preparations. Lipid droplets would

have been extracted in specimens that had been stored in alcohol. Leydig cells in some species (boar, horse) would also have contained lipofuscin pigment granules, which Leydig described as "yellow, sharply-defined point-like granules," giving a "chocolate," or "coffee-brown" color to the interstitial tissue. Leydig often recognized individual Leydig cells, as for example his Fig. 7 (our Fig. 2B), of bat Leydig cells, which shows four individual mononucleate cells, as well as four binucleate cells, or two-cell clusters. He also sometimes stated clearly that the masses between the seminiferous tubules were made up of individual cells. However, in other cases, when he couldn't discern any intercellular boundaries within a mass, as in the cat (our Fig. 2A), he was forced to consider the possibility that a mass might be an individual multinucleate cell. The closing line of the summary statement (*see* pp. 6–7) might reflect that possibility.

FURTHER 19TH CENTURY WORK ON LEYDIG CELLS

In 1854, Albert Kölliker described these cells in the human testis *(21)*, which had not been covered in Leydig's original 1850 article *(1)*. The description occurred in the male reproductive section of a two-volume advanced histology textbook entitled "Human Microscopic Anatomy or Histology" *(Mikroskopische Anatomie oder Gewebelehre des Menschen)*, in volume 2, which was devoted to organ histology. There were no figures illustrating the cells, and the description (ref. *21,* p. 392) was brief. (*Note*: the human testis are divided into 200–300 lobules, each usually, containing one to three seminiferous tubules; the thin connective tissue partitions that lie between the lobules are called septula): "As soon as the testis lobules are completely isolated, then the septula appear as effective separating partitions. These septula contain, in their loose connective tissue, many pale, round cells, similar to those found in embryonic connective tissue. In older men the cells are single or numerous, often gathered together in clusters, becoming enlarged and producing fat droplets, or brown pigment granules in their cytoplasm. Similar cells are found more sparsely among the seminiferous tubules in the connective tissue that hold the lobule together." The bibliography at the end of that chapter includes Leydig's 1850 article *(1)*, but it is only cited in the text regarding the male tract and accessory glands.

Leydig's 1857 textbook of comparative histology *(19)* contained a description of Leydig cells in the testes of various mammals, most of the information based on his article of 1850 *(1)*. The book does not include any figure that shows Leydig cells. The chapter on the vertebrate male reproductive system has a half-page summary statement about these cells, similar to the summary in Leydig 1850. The only new information on Leydig cells is that they are present in the testis of the lizard *Lacerta agilis*. At the end of the chapter, information from the 1850 article (the source is clearly stated) describing the male tract and the testis in various mammalian groups, is abstracted in small print, including some mention of Leydig cells. Although the book contains a separate chapter on the human reproductive system, there is no reference to Leydig cells in that chapter.

As the methods of specimen preparation for light microscopy improved over the latter half of the 19th century *(8)*, it was possible to observe Leydig cells in more accurate detail. Early microtomes for cutting sections came into serious use for biological investigation in the 1860s. Simple embedding of a specimen in paraffin wax for sectioning dated from 1869, but true infiltration with paraffin began about 1881. Osmium tetroxide was first described as a fixative in 1864, whereas the use of formaldehyde for fixation did not begin until 1893. The first use of two different stains on the same section (double staining) was described in 1867. Hematoxylin was first used with a mordant, allowing effective staining, in 1872. Synthetic dyes for staining sections came out in the 1860s (aniline blue, basic fuchsin), 1870s (eosin, safranin, acid fuchsin, orange G), 1880s (methylene blue, malachite green), and 1890s (toluidine blue, neutral red, azocarmine). The homogeneous oil immersion lens was introduced by Carl Zeiss and Ernst Abbé in 1878, and in 1883 Abbé completed calculations for the first apochromatic objective lens, corrected for chromatic aberration at three wavelengths and also for spherical aberration *(6)*. Utilizing these highly improved preparative techniques and optics, workers were able to provide more exacting descriptions of Leydig cells in animals of various species, at diverse stages of developmental and physiological states. A list of some of these articles is given in Table 1.

One of many examples of fine histological detail achieved during this period was Reinke's 1896 description of crystalloids in human Leydig cells *(27)*. Friedrich Reinke (1862–1919) *(10,28)* was in the Anatomical Institute of the University of Rostock, in northeastern Germany. His training included studies at the University of Kiel, where professor Flemming had stimulated his interest in histology. These Leydig cell crystalloids had been overlooked in previous histological studies of the human testis, probably because it was difficult to obtain fresh human material and because the most favorable preparative procedure for this human tissue had not yet been worked out. Reinke was fortunate to obtain fresh testis tissue from a 25-yr-old

Table 1
Studies on Testicular Leydig Cells in Various Animals (Alphabetical Listing by Common English Name and Year)[a]

Animals	*Year*
Bat	1850 *(1)*, 1923 *(118,119)*, 1925 *(120)*, 1927 *(121)*, 1939 *(122)*, 1940 *(123)*
Cat	1850 *(1)*, 1873 *(124)*, 1879 *(32)*, 1897 *(31,34)*, 1898 *(29)*, 1903 *(35)*, 1908 *(74)*, 1912 *(79)*, 1924 *(70)*, 1928 *(125)*
Cattle	1903 *(35)*, 1908 *(74)*, 1922 *(126)*, 1923 *(127)*, 1944 *(103)*
Chicken	1904 *(128)*, 1911 *(129)*, 1912 *(130,131)*, 1917 *(132)*, 1919 *(133)*, 1922 *(134,135)*, 1924 *(136–138)*, 1926 *(139)*, 1929 *(140)*
Deer *(Odocoileus, Cervus)*	1949 *(141)*
Dog	1879 *(33)*, 1901 *(142)*, 1903 *(35)*, 1911 *(129)*
Dormouse (*Eliomys*)	1911 *(129)*
Duck	1922 *(143)*
Fish	1921 *(144–146)*, 1923 *(148)*, 1924 *(149,150)*, 1925 *(151,152)*
Frog	1898 *(155)*, 1908 *(156)*, 1911 *(130)*, 1913 *(157)*, 1923 *(158)*, 1924 *(159–162)*, 1925 *(163)*
Goose	1926 *(140)*
Guinea pig	1903 *(35)*, 1904 *(164)*, 1922 *(165,166)*, 1923 *(167)*, 1924 *(71,168)*, 1928 *(169)*
Hare	1850 *(1)*
Hedgehog (*Erinaceus europeus*)	1911 *(82)*, 1925 *(121)*, 1927 *(122)*, 1934 *(170)*
Horse	1850 *(1)*, 1879 *(32)*, 1903 *(35)*, 1904 *(61)*, 1905 *(60)*, 1908 *(76)*, 1933 *(171)*
Human	1854 *(21)*, 1872 *(2)*, 1879 *(32)*, 1895 *(30)*, 1896 *(27)*, 1897 *(34,172)*, 1903 *(35)*, 1907 *(173)*, 1908 *(75,174)*, 1911 *(130)*, 1912 *(175)*, 1920 *(176)*, 1921 *(177)*, 1923 *(167,178)*, 1927 *(179)*, 1928 *(180)*, 1930 *(90)*, 1934 *(181)*, 1938 *(182)*, 1939 *(183)*, 1948 *(184)*, 1950 *(185–187)*
Jackdaw (*Corvus monedula*)	1919 *(188)*, 1921 *(189)*
Kangaroo	1879 *(32)*
Lizard	1857 *(19)*, 1911 *(130)*, 1929 *(190)*, 1930 *(191)*
Marmot (*Marmota marmota*)	1895 *(30)*, 1903 *(192)*, 1927 *(122)*
Mouse	1850 *(1)*, 1897 *(31)*, 1922 *(136)*, 1923 *(193)*, 1924 *(71)*, 1945 *(194)*
Mouse (*Peromyscus*)	1948 *(195)*
Mole (*Talpa europea*)	1850 *(1)*, 1873 *(125)*, 1904 *(196)*, 1909 *(197)*, 1911 *(198)*, 1912 *(199)*, 1925 *(121)*
Opossum (*Didelphys virginiana*)	1918 *(200)*
Pig	1850 *(1)*, 1873 *(125)*, 1879 *(32)*, 1895 *(30)*, 1901 *(45)*, 1903 *(35)*, 1904 *(73,201)*, 1905 *(74)*, 1911 *(202,203)*, 1812 *(80)*, 1921 *(204)*, 1925 *(205)*
Rabbit	1850 *(1)*, 1904 *(164,201,206)*, 1924 *(71)*, 1928 *(169)*, 1950 *(207)*
Rat	1850 *(1)*, 1871 *(208)*, 1879 *(32)*, 1900 *(209,210)*, 1919 *(211)*, 1920 *(70)*, 1922 *(136)*, 1924 *(168)*, 1939 *(212)*, 1943 *(213)*
Salamander	1913 *(157,214)*, 1921 *(215–217)*, 1922 *(218,219)*, 1923 *(220,221)*, 1924 *(161,222)*, 1925 *(223)*
Sheep	1925 *(205)*
Snake	1911 *(130)*
Squirrel	1880 *(36)*, 1908 *(75)*
Sparrow (*Passer domesticus*)	1902 *(224)*, 1943 *(225)*
Starling (*Sturnus vulgaris*)	1930 *(226,227)*
Toad	1898 *(155)*, 1911 *(130)*, 1913 *(157)*
Weasel (*Mustela ermina*)	1935 *(228)*
Widow bird (in French, *Combassou*; *Vidua*)	1923 *(229,230)*
Woodchuck (*Marmota monax*)	1917 *(83)*, 1918 *(231)*

[a]A similar but more extensive list, organized phylogenetically and including electron microscopy, is available in the author's 1975 Leydig cell review *(118)*.

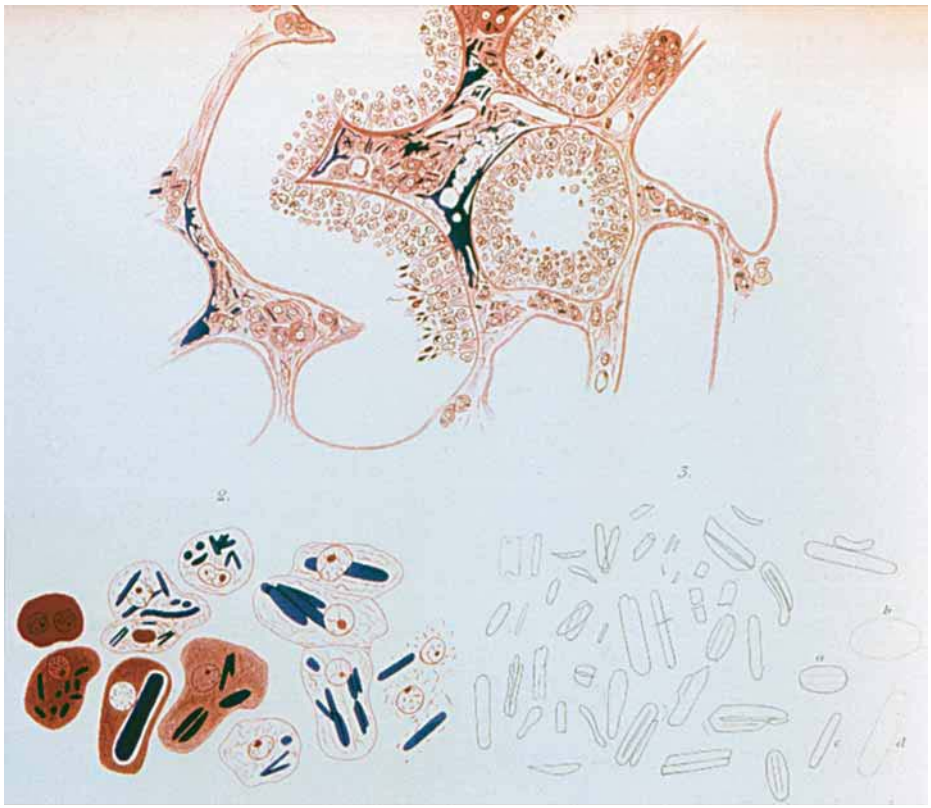


Fig. 3. Plate from Reinke's 1896 article *(27)* describing crystalloids in human Leydig cells. **(A)** Overview of human testis, showing seminiferous tubules and interstitial tissue, with Leydig cells containing crystalloids. The testis was obtained fresh from a 25-yr old executed criminal, and was fixed in absolute alcohol. Sections were stained with Weigert fibrin stain. The artist has omitted the seminiferous epithelium in some areas of the seminiferous tubules. **(B)** Detail of Leydig cells, containing the crystalloids. **(C)** Isolated, unstained, alcohol-stabilized crystalloids suspended in water, showing various forms. (Please *see* color version of this figure on color insert following p. 180.)

executed criminal, and subjected the tissue to a battery of fixatives and stains, obtaining excellent results. A copy of the plate in Reinke's article is shown here as Fig. 3. The crystalloids varied in size and in the number per cell. Having determined the best histological methods for preserving this human material, Reinke did further studies on tissue from 10 other individuals, presumably taken at autopsy, and these results were also described in the 1896 article. The crystalloids were seen in the Leydig cells of all the individuals except a 15-yr-old boy and a 65-yr-old man, neither of whom were producing sperm. A facsimile of the title and introductory paragraph of Reinke 1896, as well as a copy of the plate, have also been reproduced elsewhere *(10)*.

In the Leydig cell articles of this era there were speculations about what kind of cell type this was, as well as what function it performed. We will merely summarize some of these speculations, usually without citing specific references, because most of them were mistaken. If details are desired, they can be found in the article by Beissner *(29)*. The majority of researchers felt that Leydig cells were connective tissue cells (Leydig, Kölliker, Boll, Messing, Tourneaux, Hansemann), although others proposed that they were peripheral cells associated with lymphatic vessels (Ludwig and Tomsa, Mihalkovics), neural ganglion cells (Letzerich, Harvey), cells related to plasma cells (Waldeyer), an immature form of Sertoli cell (von Bardeleben), epithelial cells (Hofmeister), or embryonic epithelium (Nussbaum). Evidence that Leydig cells were of connective tissue nature included a study *(30)* on the male reproductive cycle in marmots, showing that the annual atrophy of Leydig cells gave rise to fibroblast-like cells in the interstitial tissue.

The most popular hypothesis for Leydig cell function during this period was that Leydig cells took up materials from the circulation, processed them, and then passed these nutrients to the adjacent seminiferous tubules to support spermatogenesis. Some workers interpreted the crystalloids that were shown by Reinke

in human Leydig cells *(27)* as a possible example of material prepared by the cells to be presented to the seminiferous tubules. Another substance that could be seen in the Leydig cells, and interpreted as evidence for this hypothesis, was fatty material (lipid droplets or lipid in secondary lysosomes), which could be demonstrated histologically by fixation and staining with osmium tetroxide. An influential article by Plato in 1897 *(31)* described the fatty material known to be present in the Leydig cells, and compared it with nearby fatty spherules seen in the base of Sertoli cells at the periphery of the seminiferous tubules. Plato showed figures from cat and mouse testes that purported to be intermediary stages in the passage of fatty material from Leydig cells to Sertoli cells, the material appearing to span the boundary layer (myoid cell layer) of the seminiferous tubules. A subsequent study by Beissner in 1898 *(29)*, saw no evidence for passage of lipid across the boundary layer in cat testis, and, in fact, most of the lipid in the seminiferous tubules was near the center of the tubules, not near the periphery.

Franz Leydig did not always get credit for discovering the cells that now bear his name. Many prominent articles on these cells in the late 1800s credited Kölliker, the most renowned histologist of the era, with the original discovery. Once the error had appeared in print, it tended to be repeated by others. Examples of influential articles that contained this mistake included Tourneaux 1879 *(32)*, Jacobson 1879 *(33)*, Hansemann 1895 *(30)*, Reinke 1896 *(27)*, Plato 1897 *(31)*, von Lenhossék 1897 *(34)*, and Bouin and Ancel 1903 *(35)*. Even if these authors had been aware of Leydig's 1850 article *(1)*, it is easy to understand how they could have overlooked its description of these new testicular cells, because the article dealt primarily with accessory glands of the male reproductive tract. The authors assumed that Kölliker's 1854 description of these cells in the human testis *(21)* was the original discovery. After crediting Kölliker, these authors usually went on to say that a few years later, Leydig, in his 1857 textbook of histology *(19)*, gave a broad description of these cells in various mammals, which of course was true. However, they missed the fact that Leydig had also given essentially the same broad description in 1850. The error was pointed out occasionally, for example by Nussbaum in 1880 (ref. *36*, pp. 85–96), and emphatically by Stieda in 1897 *(37)*, and by Beissner in 1898 *(29)*. After the turn of the century, Leydig was usually given clear priority as the discoverer of Leydig cells. Kölliker never claimed priority. The correct information was summarized succinctly by von Ebner in 1902, in the last edition of Kölliker's famous histology textbook (ref. *38*, p. 413), here quoted in translation: "Testicular interstitial cells, first described by Leydig in animals (in 1850), and by Kölliker in man (in 1854), have had a variety of functions attributed to them."

CONTROL OF MALE SECONDARY SEXUAL CHARACTERISTICS: NERVES VS HORMONES

It might seem surprising to present day readers that virtually none of the numerous articles that appeared in the 1890s on putative Leydig cell functions devoted appreciable attention to the possibility that Leydig cells served an endocrine function in the control of male secondary sexual characteristics. The concept of endocrine glands was coming into prominence during that period, because of work on the endocrine pancreas, thyroid, and other newly-discovered endocrine glands. Possible endocrine factors in the testis had actually received some notoriety in the late 1880s through the well-publicized but controversial experiments of Brown-Séquard *(39)*, a professor at the University of Paris, who injected extracts of animal testes into himself, and claimed beneficial results. However, most of the researchers who were engaged in the study of Leydig cells did not seem to associate these matters with possible Leydig cell function.

This lack of concern about testicular endocrinology was paradoxical because, as would be widely appreciated a few years later, the testis had actually been the organ in which endocrine effects were first demonstrated, back in 1849, a year before Leydig's first description of Leydig cells. This discovery *(40)*, now acknowledged as the beginning of endocrinology, was made by Arnold Adolph Berthold (1803–1861) *(41)*, of the University of Göttingen, Germany. Of course, it had been known since antiquity that castration of a male animal resulted in the reduction or loss of male sexual characteristics. Generally, at the time of Berthold's study it was assumed that the ability of the testis to maintain male characteristics throughout the body was mediated through the nervous system, with nerves from the testis being able to exert their control by way of the central nervous system. Berthold's experiments involved testicular transplantation in male chickens 2–3 mo old. When the testis of an animal was transplanted into the abdominal cavity of another animal that had been castrated, the transplanted testis was sometimes able to become established, with adequate blood supply, usually on an intestinal surface. Six months after the transplantation, these animals showed all the male sexual characteristics of roosters, including

full comb and wattle development, as well as typical rooster behavior. As Berthold pointed out, these male characteristics could not have been under the control of specific testicular nerves, because those nerves had been severed and only gastrointestinal autonomic nerves were available in the gut. However, the blood supply was well established and so the most reasonable explanation was that the testis secreted some product into the blood which was then distributed throughout the body to control male sexual characteristics. Although Berthold's study is now famous, the article was largely ignored at the time, because of initial difficulties others had in reproducing his experiments *(41)*. The concept of neural control of male secondary sexual characteristics, thus continued to be widely believed until the end of the century, which might explain why most of those who studied Leydig cells during the period were not considering an endocrine role for these cells. It was not until after the turn of the century that Berthold's work was "rediscovered" and widely recognized.

Originally, Berthold presented his experimental results in February 1849 as a talk before the Göttingen Royal Scientific Society, and the written article subsequently appeared in the proceedings of that society *(42)*. A slightly shorter version of the same article was also published that year in a more accessible journal *(40)*. Several English translations of the journal articles are available (Bremner 1981 *[43]*, for example), and a facsimile of the article from the society proceedings can be seen in Setchell's *Male Reproduction* (ref. *26*, pp. 225–229).

The possibility that Leydig cells might be involved in internal secretion was mentioned in at least two articles before 1903, the year Bouin and Ancel published their initial classic article on the subject. The first of these earlier mentions was in the 1896 article of Reinke on crystalloids in human Leydig cells *(27)*, described earlier. Commenting on the possible significance of the crystalloids, Reinke wrote (ref. *27,* p. 43): "It seems to me the most likely *a priori* that, as in the thyroid, these interstitial cells produce an unknown product, which is transported through lymph to the blood and probably carries out unknown functions. Furthermore, when this material is produced in excess, it becomes stored in the form of these crystalloids." He further suggested that "these interstitial cells, with their crystalloids, have something to do with spermatogenesis and probably with sex drive."

The second earlier mention of internal secretion was in the short 1901 article by Regaud and Policard *(44)* on pig testis. After pointing out a putative fine-granular secretory material seen in the Leydig cell cytoplasm after staining with cupric hematoxylin, and showing that the Leydig cells became abundant before seminiferous tubules had developed appreciably, the authors concluded *(44)*: "There is thus relative anatomical and functional independence between the interstitial cells and the seminiferous tubules, and these permit us to attach the secretory phenomena that is seen in the interstitial cells to a particular internal secretion that has long been suspected. The phrase "has long been suspected" might have referred to the public excitement over Brown-Séquard's experiments *(39)*, aforementioned which were highly controversial.

POL BOUIN (1870–1962) AND PAUL ANCEL (1873–1961)

Bouin and Ancel were the first to strongly emphasize a possible endocrine role for Leydig cells. They presented a well-formulated hypothesis that Leydig cells served as a gland of internal secretion to control male secondary sexual characteristics throughout the body, and they supplied extensive evidence for this hypothesis. Although their evidence was necessarily circumstantial, it was laid out beautifully in careful, detailed, and well-reasoned articles. Before describing the contributions of these pioneers in reproductive endocrinology, we will summarize their lives.

Pol André Bouin *(45–51)* was born on June 11, 1870, in Vendresse, a small village in the Ardenne region of France, near the Belgian border northeast of Reims. His father was a veterinary surgeon who had a strong interest in the physiology of domestic animals, a subject he sometimes discussed with his son, including observations on cryptorchidism. His mother was a very cultured woman who read English and German fluently. His grandfather had been a veterinarian in the imperial stables of Napoléon Bonaparte.

After completing Vendresse primary school, and receiving degrees in letters and sciences from the lycée in Charleville-Mézière (about 20 km northwest of Vendresse), in 1891 Bouin began medical training in the faculty of medicine at University of Nancy, living with relatives in that city. Two of his professors particularly caught his attention, Adolphe Nicolas, professor of anatomy, and especially Auguste Prenant (1861–1927), professor of histology. Prenant was an excellent teacher and an individual of broad erudition, who did research on seminiferous tubules. Under his inspiration, the young Bouin developed a strong interest in histology and soon began doing research on the testis in Prenant's lab, receiving a position as histological preparator *(préparateur d'histologie)* in 1892. One of Bouin's studies became his medical thesis, a project to show the effects that blockage of the ductus deferens or

epididymis would have on testicular histology. He completed his medical training, defended the thesis, and received his medical degree in July 1897. The title of his thesis was "Abnormal cytological phenomena during histogenesis and experimental atrophy of the seminiferous tubule" *(Phénomènes cytologiques anormaux dans l'histogenèse et l'atrophie expérimentale du tube séminifère)*. In an 1897 publication on experimental testicular regression *(51)*, resulting from his thesis research, Bouin described a formaldehyde–picric acid–acetic acid fixative he had found to be particularly effective for the preservation of the testis. It came to be know as "Bouin's fixative," and is one of our commonly-used histological fixatives today.

After receiving his medical degree, the young doctor was given a 1-yr position as laboratory chief *(chef de travaux)* of histology. In 1898, he successfully competed for an untenured faculty position *(professeur agrégé)* in histology and anatomy at Nancy, a 9-yr faculty appointment (1898–1907) roughly comparable with an untenured assistant/associate professorship in an American medical school today. It was during this period that Bouin, in collaboration with his friend and colleague Paul Ancel, carried out the fundamental studies providing strong evidence that testicular Leydig cells constituted an endocrine gland whose product controlled male sexual characteristics. These studies will be described below.

Bouin knew that his faculty appointment would end in 1907, and he had to decide what to do thereafter. For a time he contemplated entering into private medical practice as an ophthalmologist, which would have been a great loss to science and to academic medicine. However, in February 1907 he accepted a professorship of histology and pathological anatomy in the medical faculty of the University of Algiers, in Algeria, North Africa. A year later, Auguste Prenant, professor of histology at Nancy, left for a professorship at the University of Paris, and Pol Bouin accepted an offer to replace him in January 1908 as professor of histology at the University of Nancy. Fortunately, Adolphe Nicolas, the professor of anatomy, also left Nancy for a position in Paris at about the same time, so Paul Ancel, Bouin's friend and collaborator, became professor of anatomy at Nancy. Bouin and Ancel were thus able to continue their very productive research, but their work was now devoted primarily to the histology and endocrinology of the corpus luteum, on which they also did pioneering work.

Academic life was disrupted by World War I (1914–1918), during which Bouin served as chief of a medical service at a military hospital in Nancy. When the war ended in 1918, France regained the Alsace region at its eastern border, with Strasbourg as its capital. The Alsace had been controlled by Germany since the disastrous war of 1870, at which time the French medical faculty from the University of Strasbourg had fled west to establish the faculty of medicine at the University of Nancy. Now that Strasbourg once again belonged to France, the newly-appointed dean of the medical faculty there, M. Georges Weiss, set out to attract eminent professors from Nancy and elsewhere to re-establish Strasbourg's French medical faculty. Bouin and Ancel responded to this call and in 1919 Bouin moved to Strasbourg as professor of histology, and Ancel became professor of embryology. In recognition of Bouin's scientific stature, the Rockefeller Foundation provided funding to help him establish an outstanding modern institute of histology at Strasbourg. Bouin assembled a brilliant group of disciples, including Max Aron, Robert Courrier, Jacques Benoit, Marc Klein, Gaston Mayer, and others. During World War II (1939–1945), the University of Strasbourg was moved to Clermont-Ferrand, in central France, south of the area initially occupied by the Germans. Although Bouin had reached retirement age, he continued his duties in Clermont-Ferrand until the war ended and he returned to Strasbourg. His retirement ceremony on 18th November 1946, at Strasbourg, was attended by former associates, students, and friends from all over France and from abroad. After retirement, Bouin and his wife returned to his hometown of Vendresse. He died there on February 5, 1962, at the age of 91.

Paul Albert Ancel *(52–55)* was born on September 21, 1873 in Nancy. He studied medicine at the University of Nancy, becoming a hospital intern in 1898, and receiving the degrees of doctor of medicine in 1899 and doctor of science *(docteur ès-science)* in 1903. His doctoral thesis dealt with the development and structure of the hermaphroditic gonad of the common snail *Helix pomatia*. He served 7 yr (1897–1904) as laboratory chief *(chef de travaux)* of normal anatomy, under professor Adolph Nicolas at Nancy. During those years Ancel pursued a very productive series of collaborative studies with his friend and colleague, Pol Bouin, on the endocrine function of testicular Leydig cells. In 1904, after a broad competition for untenured faculty positions *(professeur agrégé)* at various universities, Ancel was awarded a position at the University of Lyon, under the famous professor of anatomy Léon Testut. Although the move to Lyon interrupted his fruitful collaboration with Bouin, it did allow Ancel to write an anatomy dissection guide *(56)* that became widely used. In January of 1908, Ancel returned to the

University of Nancy as the professor of normal anatomy, replacing his former mentor. Ancel resumed his collaborative research with Bouin, which now dealt mainly with the corpus luteum. In 1919, after the end of World War I, Ancel became the professor of embryology at the University of Strasbourg, the first chair of embryology in France, created especially for him. At Strasbourg his research was devoted to experimental embryology, a field that had developed rapidly in Germany but was just now beginning in France. His laboratory group came to include P. Vintemberger, Etienne Wolff, S. Lallemand (Ancel's daughter), and others. They carried out important studies on the establishment of bilateral symmetry in amphibian embryos, as well as pioneering research in experimental chemical teratology. As was described earlier for Bouin, Ancel also spent the World War II years (1939–1945) in Clermont-Ferrand. After retirement, Ancel moved to Paris, where he continued his research in the Institute of Physicochemical Biology, associated with the Collège de France. During his final years, he continued to work in a small laboratory set up in his home. He died in Paris on January 27, 1961, at the age of 87. His scientific productivity, totaling more than 300 publications, was sustained right up to the end of his life, as is indicated by two single-author articles on bilateral symmetry he published in 1960, a few months before his death.

BOUIN AND ANCEL: EVIDENCE FOR A LEYDIG CELL ENDOCRINE ROLE (1897–1905)

From 1897 to 1904, Bouin and Ancel carried out studies on testicular Leydig cells and their endocrine role in the control of male secondary sexual characteristics throughout the body. The four major articles that resulted from these studies were published in 1903 *(35)*, 1904 *(57,58)*, and 1905 *(59)*. In addition, over the same period, there were approx 19 brief articles dealing with various specific topics, appearing in the proceedings of the French Society of Biology *(Comptes Rendu de la Société de Biologie)* and the proceedings of the French Academy of Sciences *(Comptes Rendu de l'Académie des Sciences)*. These short articles represented oral presentations that were delivered before the two groups. The articles were usually two to three pages long and were not illustrated. These presentations commonly occurred in pairs, with Bouin as first author on one and Ancel first author on the other. They often enlarged on information that appeared in the main articles.

The four main articles will be summarized first, to convey some of the scope and rigor that was characteristic of the work. That summary will be followed by a description of the principal evidence that Bouin and Ancel provided for the hypothesis that Leydig cells constituted an endocrine gland, based on information presented in the main and the short articles.

The classic article published in 1903 by Bouin and Ancel *(35)*, describes Leydig cells of the pig and other mammals, both in the normal state and after experimental treatment or pathology, observed with the best histological procedures. The article is beautifully illustrated with three plates (two of them in color) containing 18 figures, and there are four additional black and white figures in the text. The article begins with a 17-page historical survey of the previous literature on Leydig cells. This is followed by a detailed 22-page description of Leydig cells in suckling and adult male pigs, illustrated with nine figures. Figure 4 in this chapter shows their first plate (their Figs. 1–7), illustrating the testes of suckling pigs. Shorter descriptions of Leydig cells in the testes of other mammals are then provided, including young and adult rabbit, guinea pig (one figure), bull (one figure) and calf (one figure), roe deer *(chevreuil)*, hare, cat (one figure and three pages of text), stallion (one figure), human term fetus (one figure), and human adult (one figure). The article ends with a 27-page discussion of the physiological significance of Leydig cells, that the cells are glandular in nature, that they are relatively independent of the seminiferous tubules, and that although they might elaborate some nutritive materials for the seminiferous tubules, they appear to constitute a gland of internal secretion to maintain sexual behavior and male secondary sexual characteristics throughout the body. Included in this section, as evidence for the functional conclusions, are further observations on the testes of cryptorchid pigs (five figures), a cryptorchid dog (one figure), as well as testes from guinea pigs (one figure) in which the testicular excurrent ducts were blocked either by ligature and resection of the vas deferens or by injection of zinc chloride into the epididymal duct. These latter observations were an extension of Bouin's earlier thesis research. A facsimile copy of the summary and conclusions from the end of Bouin and Ancel 1903 *(35)*, as well as copies of the three plates (Figs. 1–18, in black-and-white) and a fascimile of their legends is available in Setchell's *Male Reproduction* (ref. *26*, pp. 236–247).

The two 1904 articles, by Bouin and Ancel *(57)* and by Ancel and Bouin *(58)*, present a variety of experimental evidence for the endocrine role of Leydig cells. The first of these articles contains an outline of the subjects to be covered in both articles. The first article *(57)*

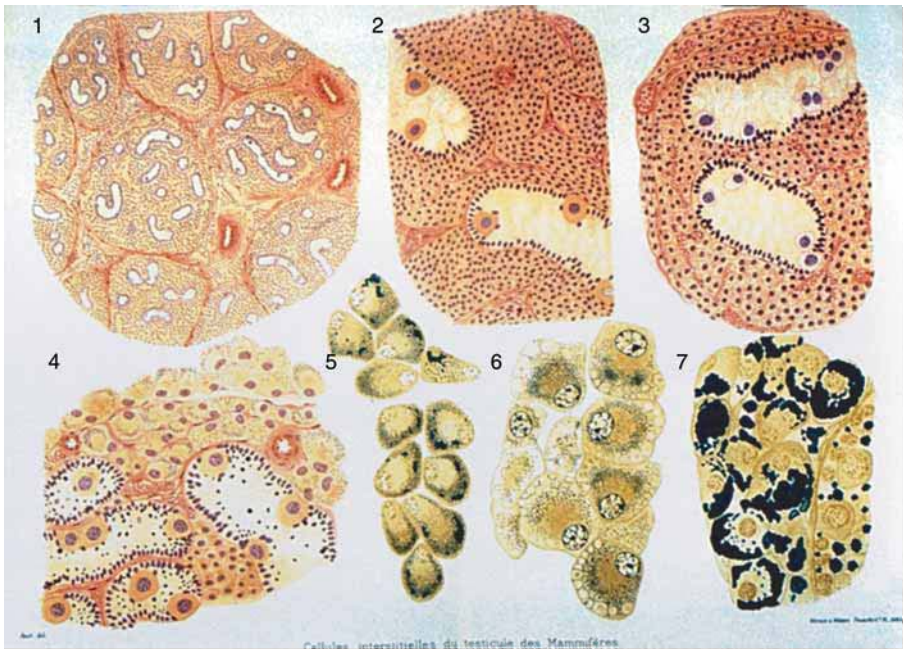


Fig. 4. First plate from Bouin and Ancel's 1903 article *(35)*, showing testes of piglets of suckling age. **(A)** Low power survey view. Several lobules are visible, each containing abundant Leydig cells and a few rudimentary seminiferous tubules (white). **(B)** Same at higher magnification, showing two seminiferous tubules surrounded by continuous masses of Leydig cells. A few large gonocytes are seen in the seminiferous tubules. **(C)** Field at the same magnification as the preceding, in the testis of a slightly older suckling pig. Note the larger size of the Leydig cells. **(D)** Still older suckling pig. The Leydig cells in the upper part of the field have become very large, their nuclei have assumed an eccentric location, and their cytoplasm is differentiated into a central region with a periphery containing osmiophilic "fine granulations" (probably lipid droplets and pigment granules) **(E–G)**. Detail of Leydig cells from the same testis shown in Fig. 4, fixed and stained in various ways. (Please *see* color version of this figure on color insert following p. 180.)

deals with evidence in adult animals that neither the germ cells nor the Sertoli cells exert any control over the male secondary sexual characteristics of the body. The experimental evidence involves cryptorchidism, blockage of the testicular excurrent ducts, compensatory testicular hypertrophy after partial castration, and observations on pathological conditions. The treatments were often given in combination. The article is illustrated with one plate, showing the testis of a normal rabbit, and the testis of a hemicastrated rabbit in which the vas deferens had been blocked: in the latter the Leydig cells appear more abundant, and the seminiferous tubules contain only Sertoli cells. The second article *(58)* deals with evidence from embryos, from immature and from old animals, utilizing experimental methods similar to those of the companion study. The very abundant development of Leydig cells in late embryos, at the time when the male tract and glands are developing, suggests the hypothesis that Leydig cells control that development, because seminiferous tubules are only rudimentary at this time. The single plate of this article shows the male tracts and accessory glands of two cryptorchid boars autopsied at about the age of 11 mo, which appear approximately normal, compared with those of a castrate animal of the same age.

The major article published by Bouin and Ancel in 1905 *(59)* provided a detailed description of Leydig cells in the horse testis throughout the life history of the animal, from the fetus through old age. The article is richly illustrated with 15 figures in three colored plates, the first plate in blue, the second in blue and yellow, and the third in red, pink, and green. The article's figures show histological views of testes in a fetus of 43 cm, colts of 4, 10, 11, 15, and 25 mo of age, and stallions of 3, 8, and 20 yr. The fetal testis in horses is remarkable for its large size (in a 7-mo fetus it can be the size of a small hen's egg, weighing 25–40 g), and for the spectacular development of the Leydig cells (fetal interstitial gland), which comprise most of its volume, because the seminiferous tubules are sparse and rudimentary. Figure 5 shows the first plate from Bouin and Ancel 1905 *(59)*, with views at very low power and high power of the Leydig cells in the fetal testis. In postnatal colts up to 9–10 mo of age, the testes are smaller (8–10 g) than fetal testes, the reduction in size resulting from atrophy of the fetal Leydig cells, the last vestiges of which are

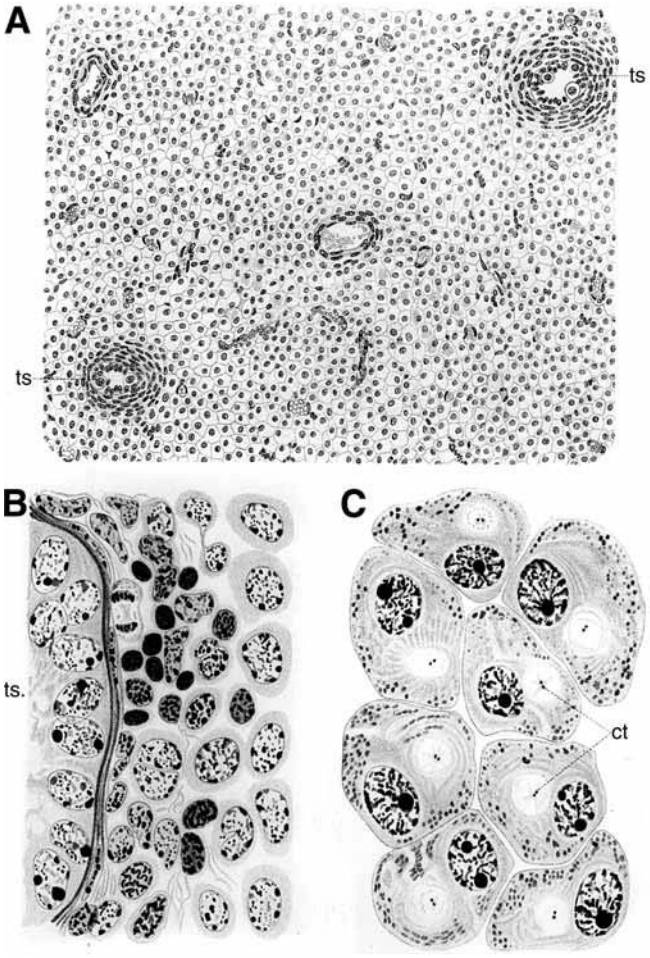


Fig. 5. First plate from Bouin and Ancel's 1905 article *(59)*, showing testis of fetal horse of 43 cm. **(A)** Low power survey view. A continuous mass of Leydig cells fills most of the field. Rudimentary seminiferous tubules are seen at lower left and upper right, and there is a blood vessel in the center of the field. **(B)** Detail, showing connective tissue (at right) alongside a seminiferous tubule (at left). The authors consider the connective tissue to be a "germinative zone" containing stages in the development of Leydig cells. **(C)** Detail of completely developed fetal Leydig cells, from the same testis seen in A and B. In each cell is seen a pale centrosome *(sphère attractive)* containing two centrioles. At the periphery of the cells are "secretory granules" *(granulations sécrétoire)*. Fixed with formol picro-acetic (now called "Bouin's fixative"), and stained with iron hematoxylin.

seen in the testes of colts 3.5 mo of age. According to the authors, a new generation of Leydig cells (young interstitial gland) arises, consisting of large cells exhibiting abundant slightly osmiophilic granules filling their cytoplasm, and with peripheral nuclei. It is likely that the authors are describing abundant macrophages, filled with secondary lysosomes, that would be engaged in cleaning up the remains of the fetal Leydig cells. The definitive adult Leydig cells (definitive interstitial gland) arise during the 2 yr of puberty required for the development of spermatogenesis in the horse testis, beginning in the 11-mo colt and reaching completion at about 36 mo. During this period, the "young interstitial gland" [made up of macrophages?] gradually disappears, its remnants persisting for a time as islands surrounded by adult Leydig cells in the interstitial tissue. Leydig cells are abundant in the adult testis, lying between the active seminiferous tubules. In older testes, the Leydig cells are smaller and less numerous. A preliminary version of the 1905 article was published in 1904 *(60)*.

The underlying hypothesis of all the work by Bouin and Ancel on Leydig cells was well expressed in their own words (ref. *61*, p. 1289), translated from French: "In numerous previous studies we have assembled a group of morphological, physiological, and chemical facts that, taken together, allow us to formulate the following hypothesis: That the general action of the testis on the organism, ascribed in the past to the testis as a whole, is actually caused by the interstitial gland." This hypothesis was applied not only to postnatal animals, but also to the embryonic development of male primary and secondary sexual characteristics (ref. *58*, p. 1041).

Listed below are the principal findings that Bouin and Ancel assembled in support of the aforementioned hypothesis, from their own studies or from the previous literature, as well as evidence they advanced against alternative hypotheses that had been previously suggested by others.

1. *Leydig cells have. the appearance of secretory cells.* Their epithelioid shape and prominent nucleus are often the morphological characteristics of cells involved in secretion. They are the only cells in the testis that clearly display this secretory morphology. Leydig cells are present throughout most of the life history in mammals and do not seem dependent on the seminiferous tubules, because they can be abundant and appear active when the seminiferous tubules are rudimentary, inactive, or atrophied.
2. *Under various experimental or pathological conditions, the seminiferous tubules regress, but the Leydig cells are still well developed, sometimes even strongly hypertrophied, and the male secondary sexual characteristics of the animal are normal.* The male characteristics are thus more closely correlated with the state of the Leydig cells than with that of the seminiferous tubules. Examples of treatments that produce these results include cryptorchidism or other heat treatments, X-rays, some cases of vasectomy, and testis grafts. The regressed seminiferous tubules that result

from these treatments usually contain Sertoli cells and some spermatogonia. However, Bouin and Ancel's extensive studies on cryptorchid pigs *(62)* yielded one animal that they claimed had abundant Leydig cells but completely lacked both germ cells and Sertoli cells *(chez l'un d'eux, cependant, le syncytium n'existait pas)*. This animal was 6.5 mo old, and had a normally-developed genital tract, indicating the presence of male hormone, apparently from the Leydig cells.

3. *During mammalian embryonic development, Leydig cells become abundant in the testis during the period when the male reproductive tract is undergoing its development, a time at which the seminiferous tubules are still rudimentary.* Consequently, any hormonal stimulation of male tract development would presumably come from the Leydig cells. A particularly striking example of the fetal development of Leydig cells is seen in the horse *(59)*.
4. *Leydig cells do not function primarily to supply nutrients to adjacent seminiferous tubules, as had been claimed by previous authors.* The supposed transfer of lipid material from Leydig cells to seminiferous tubules, described by Plato *(31)* from studies mainly on the cat testis, was not substantiated by later researchers, including Bouin and Ancel, who carefully studied the cat testis to evaluate this claim (ref. *35*, pp. 481–482). Leydig cell clusters often seem more oriented toward blood and lymph vessels than toward seminiferous tubules. Leydig cells in many species (especially cat and dog, but not in human or rat) are abundant in the mediastinum, *(1)* well away from the seminiferous tubules, which is not what one would expect if the main role of Leydig cells was to nourish the tubules.

AFTER BOUIN AND ANCEL: FURTHER EVIDENCE FOR AND AGAINST AN ENDOCRINE ROLE FOR LEYDIG CELLS

The evidence published by Bouin and Ancel in 1903–1905, that Leydig cells constituted a testicular gland of internal secretion, was extensive, although it was necessarily circumstantial because no direct evidence was available for the hypothesis that Leydig cells produced a male hormone. This situation would continue for another 50 yr, and during that time there would be further arguments in favor of the hypothesis, especially by Steinach in Vienna and by Lipschütz in Estonia, but also some compelling arguments against it, particularly from Champy in Paris and from Stieve in Halle, Germany. It is not possible here to give a detailed account of these disputes as they raged through the 1920s and into the 1930s, but it can be pointed out that by the late 1920s the balance of opinion among testicular experts probably favored the seminiferous tubule as the most likely source of male hormone. Comments will be made on some of the main issues and participants in these debates, as well as major reviews that reflected the ongoing opinion at the time they were written. These uncertainities about Leydig cell function were not finally resolved until direct evidence became available in the late 1950s that androgen was indeed produced mainly in the interstitial tissue.

The experimental procedures that Bouin and Ancel had utilized in their Leydig cell studies mainly involved blockage of testicular excurrent ducts, castration (complete or partial), cryptorchidism, and some attempts at testicular transplantation. Subsequent investigators extended these experimental approaches in new directions, and further strengthened the case that the Leydig cells were the source of a male hormone. The foremost of these workers was Eugen Steinach (1861–1944) *(63–65)*, an Austrian who worked first in Innsbruck, then in the Physiology Institute at the German University in Prague (1889–1912), and finally, in the Biological Institute of the Vienna Academy of Sciences (1912–1938). His principal results pertaining to Leydig cells appeared in 1910–1912 *(66–68)* and in 1920 *(69)*. His main approach involved castration and subsequent testis transplantation in rats and guinea pigs. Usually, the transplants were made to the inner surface of the abdominal body wall. He showed that castrates with successful testis grafts retained normal male secondary sexual characteristics. Leydig cells (which Steinach termed collectively the "puberty gland") were abundant in the grafts, and in fact constituted most of graft volume, suggesting compensatory hypertrophy. On the other hand, the seminiferous tubules always became degenerate, containing Sertoli cells but no germ cells. These studies appeared to offer strong evidence that the Leydig cells were the source of male hormone. Extending Bouin and Ancel's vas deferens ligation studies, Steinach also claimed that vasectomy could lead to a compensatory hypertrophy of the Leydig cells, and that the secretory activity of these cells could yield beneficial long-term effects, even restoring (or "rejuvenating") senile animals to the characteristics of younger animals. Another prominent worker whose studies also provided evidence for Leydig cell endocrine function was Alexander Lipschütz (1883–1980), of Dorpat University, in Tartu, Estonia. Lipschütz later summarized the field, including his own work, in an influential book published in 1924, *The Internal Secretions of the Sex Glands: the Problem of the "Puberty Gland" (70)*.

The first American to carry out extensive studies on Leydig cells was Richard Henry Whitehead (1865–1916) *(71)*, professor of anatomy and dean of the medical department, first at the University of North Carolina (1890–1905) and then at the University of Virginia (1905–1916). His Leydig cell articles, published during the period 1904–1913, dealt with fetal and postnatal development of Leydig cells in the pig *(72,73)*, detailed Leydig cell histology in various mammals *(74)*, observations on Leydig cells in crytorchidism and "hermaphroditism" *(75–77)*, and histochemical studies on fatty materials in Leydig cells *(78,79)*. His description of an unusual cryptorchid horse *(75)* strongly supported the work of Bouin and Ancel. Two testes, one normal and the other cryptorchid, had been removed from the horse, but the animal retained male characteristics and remained unruly and stallion-like. Further surgery revealed a small additional cryptorchid testis, and when that was removed, the animal assumed the appearance and behavior of a gelding. Examined histologically, the third testis contained abundant Leydig cells, whereas the only cells in the poorly-developed seminiferous tubules, were "degenerate" Sertoli cells. In contrast, Whitehead's observations on "hermaphrodite" horse *(76)* and human *(77)* both seemed to argue against Bouin and Ancel, because the body characteristics were feminine in spite of a testis containing well-developed Leydig cells. We would assume today that these "hermaphrodites" were instances of testicular feminization syndrome, resulting from a congenital deficiency of androgen receptors.

The study of seasonal reproductive cycles in various vertebrates provided results that frequently seemed contrary to an endocrine role for Leydig cells. If one postulated that Leydig cells produced a hormone that maintained male characteristics necessary for courting and mating, then one would expect Leydig cells to be most abundant at the time of the year when courting and mating occurred. However, that usually, did not prove to be the case. In many species, the time of Leydig cell abundance was distinctly out of phase with courting and mating. In some species, in fact, there were even disputes over the existence of Leydig cells in the testes. Among the workers in this field were members of Bouin's group at Strasbourg (Robert Courrier, Max Aron, and Jacques Benoit), who were favorably disposed toward the Leydig-cell hypothesis, and Christian Champy, professor of histology at the University of Paris, with whom they frequently found themselves in dispute. These extensive descriptions and discussions will not be considered here. Table 1 provides a list of studies on Leydig cells in various vertebrates. In 1928, Robert M. Oslund, of the University of Illinois, published an extensive review of vertebrate reproductive cycles *(80)*, covering fish, amphibians, birds, and mammals. Our Fig. 6 shows the graphs from that review, plotting the quantity of spermatic tissue (solid lines) and Leydig cells (dotted lines) against the months of the year. Arrows indicate the time of mating. These graphs emphasize how frequently the extent of Leydig cell development was out of phase with courting and mating. This caused Oslund to conclude that "there is no constant parallelism between sex activity and interstitial cell quantity. In fact there is in most cases an inverse relationship. These facts stand in direct opposition to the interstitial cell secretory theory" (ref. *80*, p. 266). "However, there is, one parallelism that is constant in all species… in all vertebrates the germinal tissue is maximum in quantity at the time of mating" (ref. *80*, p. 268). This suggested that some "substance produced by the metabolic processes" involved in spermatogenesis might act as the endocrine agent. The findings on vertebrate reproductive cycles had a major effect on public opinion among reproductive biologists of that era, and raised serious doubts that Leydig cells were the only or even the main source of male hormone. They suggested, rather, that the hormone probably came from the seminiferous tubules.

In Oslund's review *(80)*, described earlier (Fig. 6), only the annual cycle of the hedgehog, from the article presented in 1911 by Marshall *(81)*, of Cambridge University, appeared as one would expect if Leydig cells controlled the male characteristics for courting and mating. There was partial correspondence in the woodchuck cycle, based on the study done in 1917 by Rasmussen *(82)*, of Cornell University. Here the increase in Leydig cells indeed began before mating, but the greatest abundance of these cells occurred during and after the time when the young were born.

The various lines of observational and experimental evidence provided by Bouin and Ancel, by Steinach and by others in favor of a Leydig cell endocrine role received a great deal of criticism over the years. The evidence was all circumstantial. The various experimental treatments that caused degeneration of the seminiferous tubules, for example, suffered from an inability to eliminate Sertoli cells, leaving the possibility that those cells might actually produce the hormone. Most of these procedures also allowed the survival of spermatogonia. Claims of Leydig cell "compensatory hypertrophy" suffered from difficulty in showing that the Leydig cells actually increased, rather than merely appearing more developed because of tubule atrophy.

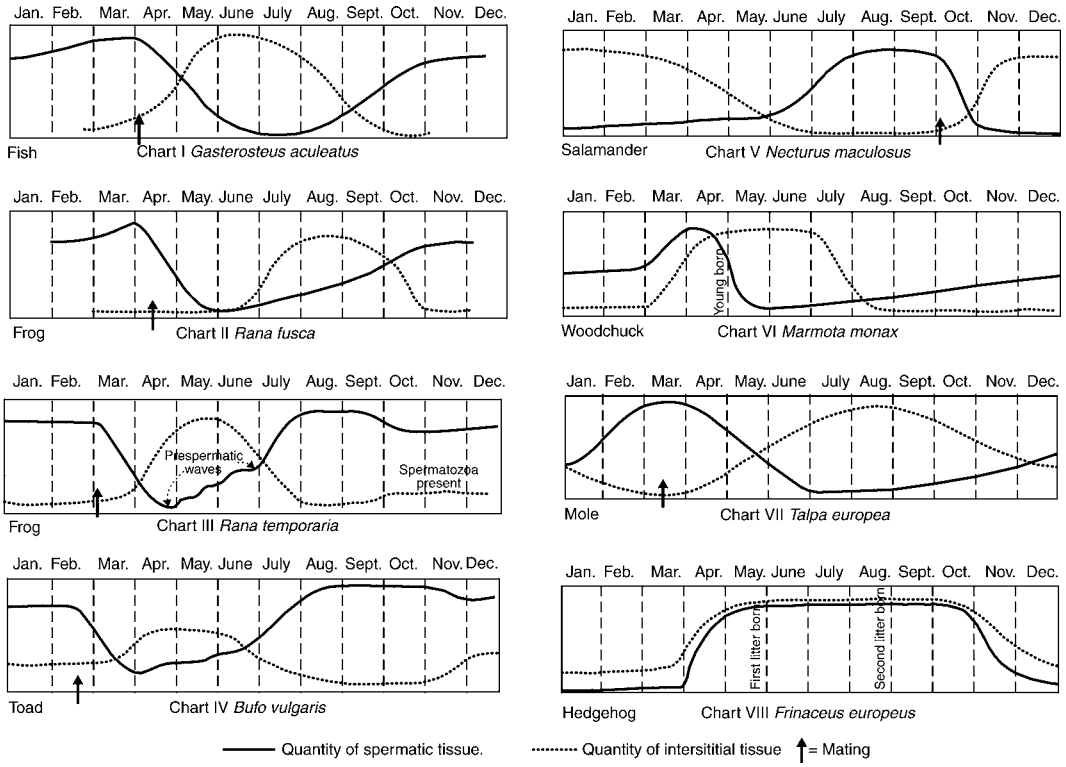


Fig. 6. Graphs from Oslund's 1928 review of vertebrate annual reproductive cycles *(80)*. Each graph compares the quantity of spermatic tissue (solid line) and the abundance of Leydig cells (dotted line) over the seasonal cycle of various vertebrates. Arrows indicate the breeding period. The times of maximal Leydig cell development usually do not correspond to the breeding periods. In the article the graphs are arranged singly or in pairs throughout the text, but are here organized into a single figure.

These and other criticisms of the Leydig cell evidence were summarized in a major review *(83)* on the biology of testis and scrotum published in 1926 by Carl R. Moore, of the University of Chicago, who was one of the prominent figures in male endocrinology in 1920s and 1930s. In a portion of his review dealing with the source of testicular internal secretion (ref. *83*, pp. 36–43), he first carefully summarized the history of Leydig cell studies, and then laid out the main points of evidence that had been provided by Bouin and Ancel, Steinach, and others that Leydig cells served an endocrine function. "But if we examine critically the main points of contention it will become apparent that the question is by no means definitely settled" (ref. *83*, p. 40). He maintained that Steinach's studies were flawed because more recent work on vas deferens ligation in various species had consistently failed to show appreciable degeneration of seminiferous tubules or hypertrophy of Leydig cells, and so Moore offered other explanations for the "rejuvenation" experiments. Moore also went over problems in interpreting the compensatory hypertrophy studies of Bouin and Ancel, as well as the fact that in vertebrate seasonal cycles the maximum development of Leydig cells was frequently out of phase with the breeding season. It is impossible here to review the many topics that were discussed, and so the interested reader is referred to Moore's review for further details. He concluded (ref. *83*, p. 43): "In general there appears a decline from the pinnacle to which the significance of the interstitial cells has been elevated in the last score of years, and at present there are those who strongly proclaim that such cells have absolutely nothing to do with determining or sustaining the secondary sexual characteristics." Other components of the testis "have in turn come in for discussion as the probable source of the internal secretion," such as germ cells ("some cells of which invariably remain after degeneration"), and the "vast Sertoli reticulum."

In 1930, 4 yr after Moore's review, described earlier, in which he was critical of the Leydig cell hypothesis, Moore published a research article *(84)*, which included an incidental finding, providing striking evidence that the interstitial tissue was indeed the source of the male hormone. The main topic of the article was the use of electrically-induced ejaculation in guinea pigs as a bioassay for the testicular hormone, the weight of the ejaculate serving as a measure of hormone level. In one

of the animals (ref. *84*, pp. 51,52), a testis had been removed and the remaining testis rendered cryptorchid 8 mo before the bioassays were carried out. The testis was autopsied about 4 mo after the bioassays. Although the weight of the single cryptorchid testis had declined to only 2% that of two normal testes, the animal had given ejaculations comparable in weight to those of a control animal, indicating a normal level of hormone. Histological examination showed that the seminiferous tubules were atrophied and devoid of germ cells, and although Sertoli cells were present, "some of the tubules were so degenerate that only their bare outlines were visible." It was "evident that this hormone was not manufactured by germ cells, for the animal had not produced germ cells for 8 mo or longer. The interstitial tissue is the most evident of the remaining mass and is probably the source of the hormone, but whether it is produced by the cells of Leydig or by other elements present in the intertubular spaces is unknown." In spite of this compelling evidence, in 1939 Moore wrote a 98-page review, "Biology of the Testis" *(85)* for the prestigious book *Sex and Internal Secretion*, without any comment about where in the testis the male hormone was produced. Perhaps he still considered the issue unsettled.

Another influential researcher on the testis in the 1920s was Hermann Stieve (1886–1952) *(86,87)*, professor of anatomy at the University of Halle-Wittenberg, in Germany. He published many articles on Leydig cells, including a 249-page treatise in 1921 on their development, structure, and significance *(88)*. Stieve was later chosen by von Möllendorf to write the male reproductive section *(89)* for volume 7 (1930) of the monumental *Handbuch der mikroskopischen Anatomie des Menschen* (Handbook of Human Microscopic Anatomy). In this 387-page section, Stieve included a thorough review of research findings on Leydig cells. He maintained that the best evidence pointed to the seminiferous tubules as the primary site of testicular hormone production, and that the role of Leydig cells was merely to nourish the tubules. He summarized this position (ref. *89*, p. 75, translated from German): "On the significance of Leydig cells I can be brief. For myself, I have the same point of view now as before that the Leydig cells constitute a nutritive support tissue of the testis, and that this interpretation can bring together all the available observations that have been made on these cells. The view that has been put forth by Bouin and Ancel, and especially by Steinach and his school, that the Leydig cells produce a sex-specific hormone, is devoid of any factual basis" *(entbehrt jeder tatsächlichen Grundlage)*.

The tide of opinion against Leydig cells as the main source of male hormone seemed to reach a peak in the late 1920s. One can still see reflections of it in Andrew T. Rasmussen's extensive 1932 review of Leydig cells *(90)*. On vertebrate seasonal cycles: "On the whole, therefore, there is too much variability to permit any rigid generalizations on the relation of the interstitial cells to the secondary sex manifestations, although in many cases there exists a close parallelism. Histological studies can never settle these questions independent of physiological and biochemical data. The parallelism may be entirely secondary and not necessarily evidence of causal relationship" (ref. *90*, p. 1694). Conclusion on Leydig cell function: "Whereas investigations of sex reversal, sex intergrades, antagonism between sex organs, and so on, have yielded many suggestive facts, the significance of the interstitial cells must still be deduced from inconclusive data and great reserve is therefore necessary in making generalizations" (ref. *90*, p. 1710).

CHEMICAL CHARACTERIZATION AND PHYSIOLOGY OF MALE REPRODUCTIVE HORMONES IN MAMMALS, INCLUDING HUMANS

After 1930 the tide began to shift back to Leydig cells as the source of male hormone, although there were still uncertainties. The 1930s saw rapid advances in our chemical understanding of the male hormone, its action in the male system, and its regulation by the pituitary gland. By 1931, the male sex hormone had been shown to be a steroid, and 15 mg of one of its forms, androsterone, had been crystallized from 25,000 L of human male urine *(91)*. By 1935 the main testicular form of androgen had been isolated from bull testis extracts, and named "testosterone" *(92)*. Testosterone's principal effects in the male system were elucidated over the next few years (review *[85]*). The synthesis and metabolism of androgen was also studied. The role of pituitary luteinizing hormone (LH, also called interstitial cell stimulating hormone [ICSH]) on testicular function in rats was characterized in 1936 by Greep, Fevold, and Hisaw at Harvard *(93)*, and in 1937 by Evans, Simpson, and Pencharz in Berkeley *(94)*.

Most of the fundamental studies on androgen biosynthesis, metabolism, and physiology were originally carried out in domestic or laboratory mammals. However, comparable investigations in the human male were done eventually, primarily in the 1960s. These early human studies have been reviewed by Bardin *(95)*. Although it was assumed that testosterone was the

main androgen in human males, it was initially difficult to measure it in plasma or urine. By 1960, new methods for the quantitative chemical analysis of steroids became available, involving various types of chromatography (paper, thin layer, gas) and the use of steroids labeled with isotopes (^{3}H and ^{14}C) of high specific activity. Using these new methods, it was possible to show accurately what androgenic steroid hormones were present in the human testis, plasma, and urine. Levels of testosterone were measured in male blood plasma *(96–99)*, and it was shown that testosterone was secreted by the testis, because the concentration was higher in testicular vein blood *(98,100–102)*. Subsequent work determined which of the androgenic steroids played a primary role in stimulating the testis, male tract, and other target tissues, and to recognize precursors and metabolites. It was also important to determine the comparative role of testis and adrenal in these steroid interactions. By the end of the 1960s, the fundamental pattern of androgen endocrinology in the human male had been fairly well worked out.

The new chemical understanding of the mammalian male hormone, its actions and its regulation, did not settle the issue of where the hormone was produced in the testis, but it at least provided some additional evidence in favor of the Leydig cell. For example, the injection of gonadotropin would only enhance male secondary sexual characteristics if the Leydig cells were visibly stimulated *(93,94)*. However, when World War II (1939–1945) brought this era of rapid advance in male endocrinology to a close, there was still some uncertainty about the site of androgen production in the testis, and still no direct evidence that Leydig cells produced the hormone.

One of the observations that continued to argue against androgen production by Leydig cells was the lack of appreciable change in the number or appearance of these cells during puberty, when the male secondary sexual characteristics were undergoing dramatic development. The seminiferous tubules, on the other hand, were involved during puberty in the establishment of spermatogenesis, a prominent activity which suggested to many workers that cells of the tubules were more likely to be the source of male hormone. The situation was comparable with vertebrate seasonal cycles, where the abundance of Leydig cells frequently seemed out of phase with the characteristics they were supposed to be controlling. In 1944, Charles W. Hooker, of Duke University, published an important article *(103)* dealing with this issue. The article began with the statement: "After 40 yr of study, the evidence that the Leydig cells secrete androgen is for the most part unsatisfactory." The study was carried out on 30 bulls, ranging in postnatal age from 1 mo to 15 yr. Each testis was weighed, the total androgen content per testis was measured by bioassay, and a tissue sample was subjected to careful histological study. The goal was to determine which testicular component, Leydig cells or seminiferous tubules, correlated best with testicular androgen content over the life history of the animal. The results included a graph showing testicular weight and androgen content plotted against animal age. Testicular androgen content increased at a low, continuous rate over the first 2 yr, during which the curve for testicular weight increased more rapidly. There was no striking change in Leydig cell morphology or in testicular androgen content during puberty, which occurred during 6–12 mo. This suggested an increase in target organ sensitivity to hormone during puberty. The dramatic increase in seminiferous tubule size during puberty was not associated with any change in testicular androgen level. Androgen content increased more rapidly after 2 yr, possibly correlated with the appearance of lipid droplets (vacuolation) in the Leydig cells. The article was considered strong, though indirect evidence that Leydig cells and not the seminiferous tubules, were the source of testicular androgen.

Roy O. Greep, codiscoverer of LH action on the testis *(93)*, wrote a chapter on the male reproductive system for a histology textbook he edited in 1954 *(104)*. "That the interstitial cells are responsible for secretion of the testicular androgenic hormone is widely accepted. The histochemical evidence has, for the most part, added conformation to the belief that the interstitial cells are the secretory elements of the testis" (ref. *104,* p. 761). The histochemical evidence to which he referred came from two articles that had appeared in 1942 *(105)*, and in 1949 *(106)*, utilizing a histochemical approach that was thought to allow the direct localization of androgens in the testis. A figure from one of the articles was published in Greep's chapter, showing striking localization of activity in the interstitial tissue. However, in 1955, a year after Greep's chapter appeared, the histochemical method was shown to be nonspecific *(107,108)*. The 1957 edition of the classic histology textbook of Maximow (who had done early Leydig cell research) and Bloom accurately summarized the situation (ref. *109,* p. 490): "Some authors ascribe the production of testosterone to the interstitial cells; others, to the seminiferous epithelium (spermatogenic and Sertoli cells). A third possibility is, of course, the participation of both elements. Most of the data favor the first hypothesis." The authors then went on to review the substantial but indirect evidence that Leydig cells produced testicular androgens.

DIRECT HISTOCHEMICAL EVIDENCE

The Steroidogenic Enzyme 3β-Hydroxysteroid Dehydrogenase Is Localized Mainly in Leydig Cells of Rabbit Testis (1958)

The first direct evidence that testicular androgen was produced primarily in Leydig cells was achieved with a histochemical technique that could localize the steroidogenic enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) in the testis. The article *(110)* was published in 1958 by Lee W. Wattenberg, of the Department of Pathology at the University of Minnesota School of Medicine.

In the late 1940s, while he was a medical student at Minnesota, Wattenberg had developed a strong interest in histochemistry through a research project he pursued in the laboratory of David Glick, one of the outstanding histochemists of the era. After receiving his M.D. degree in 1950, Wattenberg accepted a position in the Department of Pathology at Minnesota and began a research program localizing enzymes by histochemical methods, with special emphasis on applications to pathology. He became particularly interested in the localization of dehydrogenases, and was one of the pioneers in the use of tetrazolium salts for this localization. He decided to apply this method to the enzyme 3β-HSD, a critical dehydrogenase in the biosynthesis of all steroid hormones. In the tetrazolium procedure, the hydrogen that became available during dehydrogenation was transferred to a tetrazolium salt (nitroblue tetrazolium, neotetrazolium), a dye that was colorless and soluble in the oxidized state, but formed an insoluble, dark blue precipitate (called formazan) when it was reduced. The reaction also required an appropriate 3β-HSD substrate (dehydroepiandrosterone), nicotinamide, NAD^+ and the presence of an endogenous NADH diaphorase, necessary for transfer of the hydrogen from NADH to the tetrazolium. The dark, insoluble formazan product in the sections was found in cells that contained 3β-HSD, thereby localizing potential sites of steroid hormone biosynthesis. Wattenberg's 1958 article *(110)* applied the method to adrenal cortex, ovary, and testis. Our Fig. 7, from his article, illustrates a 3β-HSD histochemical localization carried out on a cryostat section of rabbit testis, showing clear-cut activity in cells of the interstitial tissue.



Fig. 7. Figure from Wattenberg 1958 *(110)* showing a cryostat section of rabbit testis stained histochemically for the steroidogenic enzyme 3β-HSD. The demonstration of activity for this enzyme (dark stain) in the Leydig cells was the first direct evidence that these cells were the source of testicular androgen. The section was stained histochemically with neotetrazolium, and counterstained with light methyl green. The figure has been enlarged here to almost twice its original size, and is included in this chapter with permission from Dr. Wattenberg.

DIRECT BIOCHEMICAL EVIDENCE

Androgen Biosynthesis Is Much More Active in Isolated Interstitial Tissue Than in Isolated Seminiferous Tubules of the Rat Testis (1965)

The first direct biochemical evidence that testicular interstitial tissue was the main source of androgens, published by Christensen and Mason in 1965 *(111)*, was based on the finding that seminiferous tubules could be pulled out of the interstitial tissue in pieces of rat testis. This allowed the seminiferous tubules and interstitial tissue to be incubated separately with a radioactive steroid precursor, and the biosynthetic products could then be analyzed by steroid biochemistry. Christensen had happened onto this method of separation, when he was a biology graduate student at Harvard in the 1950s. A few days after receiving his PhD in 1958, he tried a biochemical incubation run with separated seminiferous tubules and interstitial tissue, under the guidance of a steroid biochemist, Herbert H. Wotiz, from the

Biochemistry Department of Boston University School of Medicine, who had been collaborating with Frederick L. Hisaw, one of Christensen's PhD mentors. The run was carried out in Dr. Hisaw's lab at Harvard, and ^{14}C-labeled sodium acetate was used as the steroid precursor. Dr. Wotiz arranged for the steroid analyses to be done by Norman R. Mason, who was working with Kenneth Savard, in the Department of Medicine at the University of Miami School of Medicine. The results were negative, with no evidence of androgens in the incubates of either the interstitial tissue or the seminiferous tubules, presumably because sodium acetate, an extremely early precursor, had been utilized.

A few years later, when Christensen was an assistant professor in the Anatomy Department at Stanford University School of Medicine, it occurred to him to repeat the separation experiment, this time utilizing a later precursor, progesterone. He contacted Dr. Mason to find out if he was still willing to analyze the steroids, to which he agreed. The separation of testis components and the incubations with ^{14}C-labeled progesterone were carried out at Stanford, following detailed notes that had been made during the earlier incubation run with Dr. Wotiz. When the runs were completed, the incubation media were shipped to Dr. Mason for steroid analyses. This time the results were positive. Analysis of the ratio of radioactivity of products isolated from the incubation medium from interstitial tissue incubations to that from the seminiferous tubules was found to be 13.5 for 17-hydroxyprogesterone, 6.9 for Δ^4-androstenendione, and 3.5 for testosterone. These results showed that most of the enzyme activity for converting progesterone to testosterone resided in the interstitial tissue. Figure 8, taken from the article, compares the appearance of isolated interstitial tissue with that of isolated seminiferous tubules.

LEYDIG CELLS IN VARIOUS VERTEBRATES

As has been described earlier in this chapter, many experts in the 1920s and 1930s were convinced that vertebrate Leydig cells were not the source of testicular hormone, even in laboratory mammals. However, direct evidence was eventually, obtained by histochemistry in 1958 *(110)* and by biochemistry in 1965 *(111)* that Leydig cells did indeed produced testicular androgen in laboratory mammals. Since then many studies in other vertebrates (including nonlaboratory mammals) have shown that Leydig cells are the main source of androgen in the testes of most major vertebrate groups. A description of these studies is included in a 1996 review of the comparative cytology of vertebrate Leydig cells by J. Pudney *(112)*. The principal conclusions of that review will be summarized here (see the review for further details).

Modern research on vertebrate Leydig cells has extended our understanding to many more species than were covered in older studies, has applied newer methods to them, and has also provided deeper insight into the details of testicular organization and the complexities of seasonal cycles. Researchers have been able to use modern methods to recognize cells that produce steroid hormones such as androgens. One of these methods is the histochemical localization of 3β-HSD *(110)*, an essential enzyme of steroid hormone biosynthesis. This method has been very useful, even though it requires frozen sections in which the structural detail is usually not very well preserved, often making it difficult to identify cells clearly. Another approach is to use the electron microscope (EM) to examine the cytoplasm of cells for features that are characteristic of steroid secreting cells, such as an abundant smooth endoplasmic reticulum (SER), as well as tubular cristae in mitochondria (rather than the usual lamellar cristae).

These various methods have now been applied to diverse vertebrate groups, including fish (lampreys, hagfish, sharks, rays, and bony fish), amphibians (newts, salamanders, frogs, and toads), reptiles (turtles, lizards, snakes, crocodiles, and alligators), birds, and mammals. Although the evidence varies in depth and confidence, it appears that Leydig cells are present in most of these groups and that there is usually evidence that the cells produce androgens. As described earlier, it was possible in rat testis to separate seminiferous tubules from the interstitial tissue, allowing the isolated components to be tested biochemically for androgen biosynthesis *(111)*. Unfortunately, this means of localizing androgen production is not possible in most other vertebrates (or even in other mammals) because of difficulty in carrying out the tissue separation. However, Pudney et al. *(113)* have successfully used this method to localize androgen biosynthesis in the testis of a ground squirrel, *Citellus lateralis*. Androgen was synthesized primarily in the interstitial tissue, and examination of the Leydig cells by EM showed abundant SER in their cytoplasm *(114)*.

The finding that Leydig cells are the predominant source of testicular androgens throughout the vertebrates does not eliminate the possibility that Sertoli cells might also provide hormone, either in addition to Leydig cells or even in place of them. In the dogfish shark *Squalus acanthias* (Chondrichthys), the Leydig cells are very poorly developed, whereas Sertoli cells

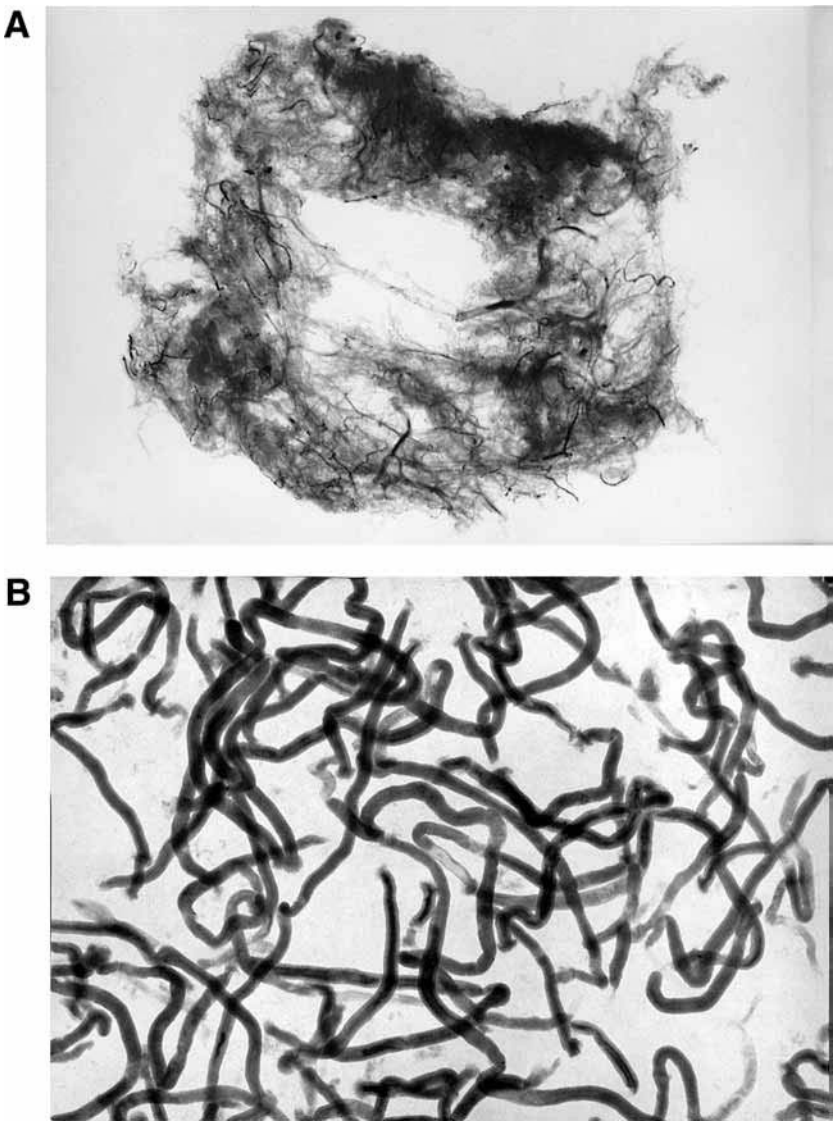


Fig. 8. Figure from Christensen and Mason 1965 *(111)* comparing isolated interstitial tissue **(A)** with isolated seminiferous tubules **(B)**, both at the same magnification. The seminiferous tubules had been manually pulled out of the interstitial tissue from a piece of rat testis, using jeweler's forceps. When the two components were incubated separately with radioactive progesterone, most of the conversion of this substrate to androgens occurred in the interstitial tissue incubate, providing the first biochemical evidence that this tissue was the principal site of androgen production.

are large and prominent during spermatogenesis. The Sertoli cells show a positive reaction for 3β-HSD *(115)*, and their cytoplasm contains abundant SER when viewed by EM *(116)*. This suggests that Sertoli cells are the primary source of testicular androgen in these fishes. The largest group of fish, the bony fish (Osteichthys), includes an extremely large number of species which show considerable diversity in testicular organization and in breeding patterns. Many of these fish have been shown to have Leydig cells, but the majority have yet to be investigated, and so there could be surprises.

And so the cells that were discovered by Franz Leydig, and for which an endocrine function was proposed and studied by Bouin and Ancel, are now well established as the primary source of testicular hormones throughout the vertebrates. The male endocrinology of nonmammalian vertebrates is reviewed in Chapter 15.

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During the writing of this chapter, I have benefited from previous reviews and historical studies, especially those of Setchell *(26)*, Moore *(83)*, Rasmussen *(90)*, Beissner *(29)*, and from the historical account that was

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II CELL BIOLOGY

2 Structural and Histological Analysis of Leydig Cell Steroidogenic Function

Syed G. Haider, PhD, Gisela Servos, and Nicole Tran

SUMMARY

This chapter summarizes the morphological criteria to identify the fetal and adult Leydig cells in the rat testis at the light and electron microscopic levels. LH receptor and 3β-hydroxysteroid dehydrogenase are the most important functional markers that can be used to characterize the androgen-producing Leydig cells. The focus of this chapter is on the structure, ultrastructure, steroidogenic function, and ontogenesis of fetal and adult Leydig cells.

Key Words: Fetal and adult Leydig cells; mammalian testis; ontogenesis; ultrastructure.

Two different populations of Leydig cells can be recognized in the testis of rodents: (1) Fetal Leydig cells (FLCs) and (2) Adult Leydig cells (ALCs). These two populations are different in topography, structure, ultrastructure, life span, capacity for androgen synthesis, response to antiandrogens, and mechanism of regulation by pituitary gonadotropins and growth factors *(1–3)*. The following description of Leydig cells is based on studies of the rat testis because these species have provided the most information.

FETAL LEYDIG CELLS

Histology of FLCs

The FLCs can be recognized at light microscopic level from *fetal day* (FD) 14.5 to *postnatal day* (PND) 25, identified by the presence of 3β-HSD (hydroxysteroid dehydrogenase) and luteinizing hormone (LH) receptors. The FLCs are arranged exclusively in clusters in the interstitial space between seminiferous tubules. A typical FLC cluster shows the following histological features: the cluster contains large round or oval-shaped Leydig cells with a round conspicuous nucleus with very often a nucleolus (Fig. 1A,B). Rarely, a mitotic figure of the nucleus is also observed in a cluster cell. All clusters are surrounded by a thin sheath of fibrocytes that are connected to one another by cell processes. Collagen fibers (CF) are present in various abundance between the FLCs within a cluster. In most cases the clusters lie in close vicinity of interstitial blood capillaries.

The FLCs clusters change their size and shape during the period between FD 15 and PND 25. In the beginning, the clusters are very large and irregular in shape occupying nearly the whole interstitial space, left by testicular cords. The formation of testicular cords begins on FD 14.5 at the cortex of the testis and progresses gradually toward the center, and finally to the hilus near the mesonephros. The temporal coincidence of the formation of testicular cords and the differentiation of FLCs in the fetal testis suggests that the peritubular cells contribute to trigger mechanisms that initiate the FLCs differentiation via paracrine factors *(4)*. The growth peak of FLCs is observed on FD 19 *(5)*. Thereafter, for example, on PND 1, the large complexes are scattered into many small clusters that occupy the interstitial space. After that the clusters show the typical histological features mentioned earlier (Fig. 1). The number of FLCs clusters decreases gradually from PND 5 onward, so that only 5–7 clusters are observed in a testicular cross-section on PND 10 and nearly 3–4 clusters on PND 15. Only one or rarely two clusters are present on PND 25.

Ultrastructure of FLCs

Table 1 summarizes the main ultrastructural features. The oval-shaped FLCs precursors contain smooth endoplasmic reticulum (SER), mitochondria

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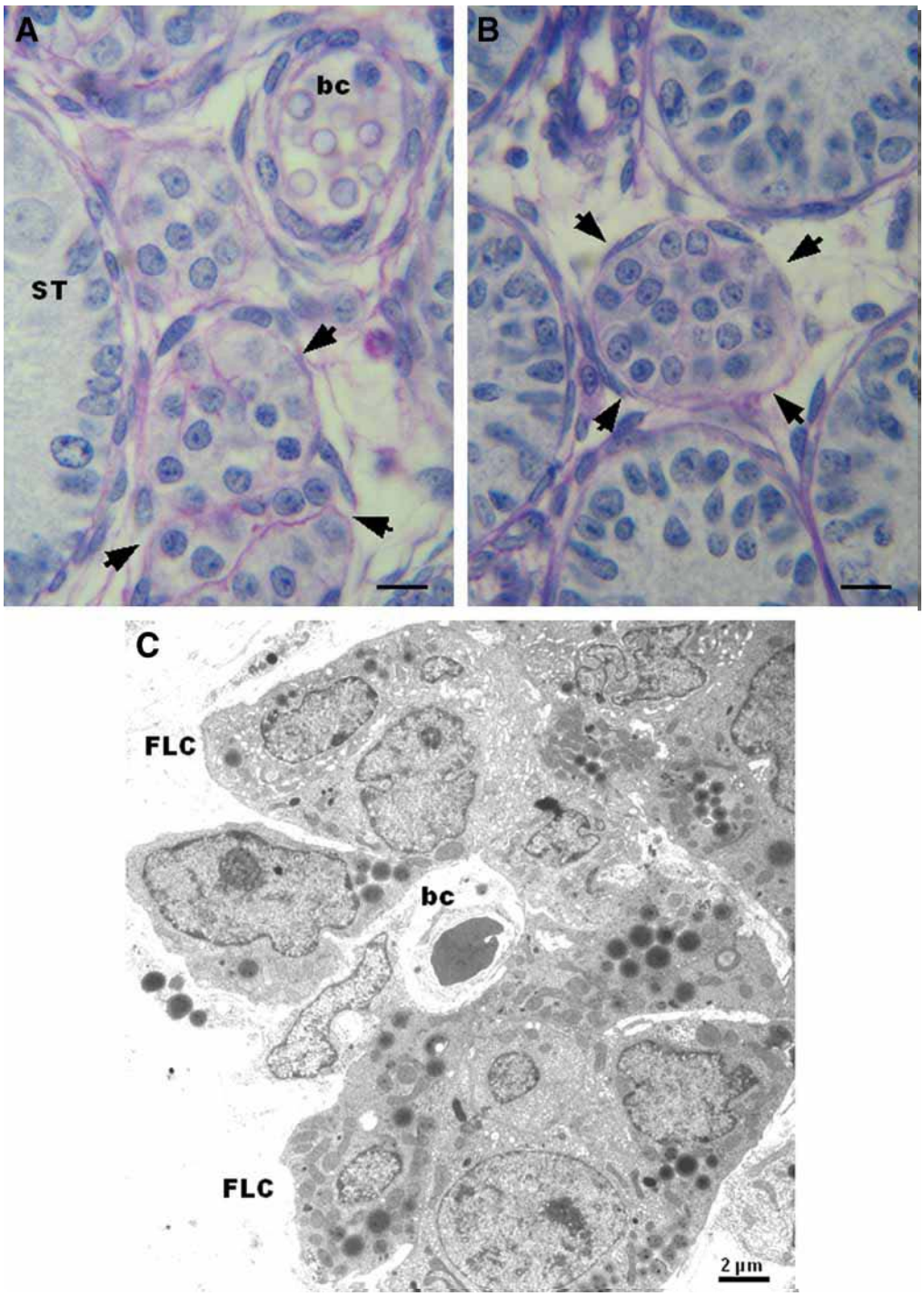


Fig. 1. FLCs in rat testis. **(A**,**B)**: bar = 10 µm; PAS (violet-red) with hematoxylin (blue) staining, age PND 5. **(A)** Three arrows (◄) point to a cluster of FLCs in the interstitial space between seminiferous tubules (ST). The nuclei of Leydig cells are large and round with a nucleolus. The thin violet-red lines in and around the FLC cluster indicate the patchy basement membrane. The cluster is surrounded by fibrocytes with thin slender-shaped nuclei and blood capillary (bc). **(B)** Four arrows (◄) surround a cluster of FLCs; thin slender-shaped nuclei of the fibrocytes at the periphery of the cluster. **(C)** Ultrastructure of FLCs building a cluster around a bc on PND 1. (Please *see* color version of this figure on color insert following p. 180.)

of tubulo-vesicular type, and small lipid droplets (Fig. 1C). They are clearly distinguishable from normal mesenchymal fibroblasts, which are spindle-shaped, with elongated nuclei, rough endoplasmic reticulum (ER), and do not contain tubulo-vesicular mitochondria, SER or lipid droplets. From FD 16 onward, the FLCs are large, round with a round nucleus, a moderate-sized Golgi apparatus, abundance

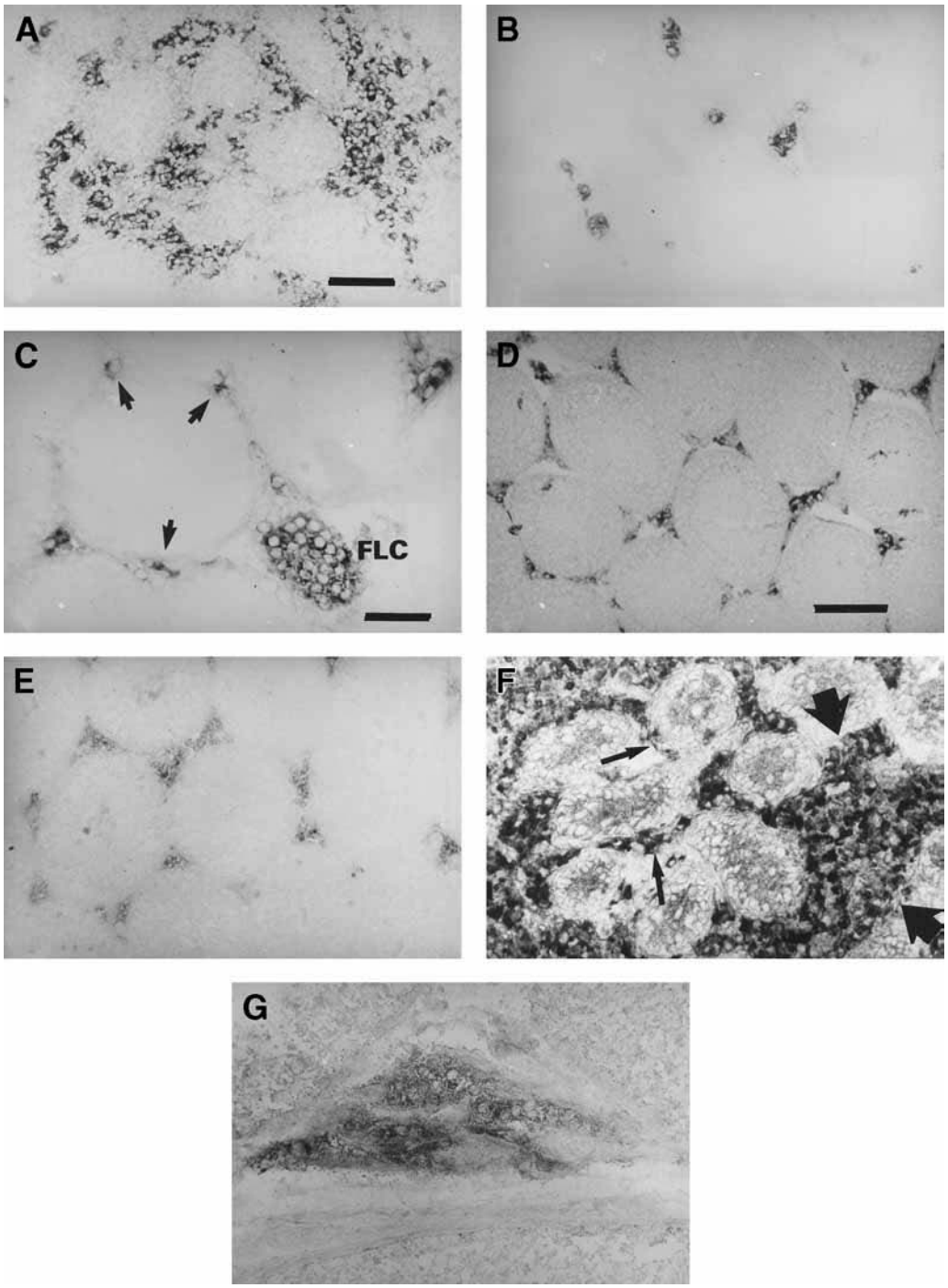


Fig. 2. 3β-HSD histochemical reaction in the Leydig cells in rat **(A–D)**, mouse **(E)**, pig **(F)**, and bull **(G)** testis. (Bar = 50 μm for figure parts **A**,**B**,**F**,**G**; 40 μm for figure part (**C**); and 100 μm for figure parts (**D**,**E**). **A** The reaction product is located in the large complexes of FLCs on fetal day 17. (**B**) FLCs cluster on PND 3. (**C**) One fetal Leydig cell cluster (FLC) and progenitors of ALCs (arrows) on PND 20. (**D**) Immature ALCs on PND 35. (**E**) adult mouse testis. (**F**) adult pig testis with the reaction in intertubular (thick arrows) as well as in peritubular (thin arrows) Leydig cells. (**G**) Leydig cells in an interstitial triangle of an adult bull testis.

of SER, mitochondria and many large lipid droplets (average diameter 0.9 μm; Figs. 1C and 3). The cell membranes of FLCs possess numerous characteristic flat, finger-like, polyhedral, and interdigitating protrusions. These protrusions contain cytoskeletal filaments and show a constant thickness of 50–60 nm, with a 25-nm-wide space between the membranes of two adjacent protrusions *(3)*. Cell contacts are observed between adjacent FLCs; and between FLCs and the neighboring vascular endothelial cells. Special cell

Table 1
A Comparison of Ultrastructural Features of Fetal and Adult Leydig Cells

Features	*In fetal LC*	*In adult LC*	*Common in both types*
Nucleus	Round with smooth membranes	Elliptic or round with curly membranes	Thick euchromatin, numerous nuclear pores
SER			Present in abundance
Rough ER	Largely absent	Only a few	
Mitochondria			Tubulo-vesicular cristae
Golgi apparatus	Small to moderate size	Large and well differentiated	
Lipid droplets	Numerous (diameter = 0.9 μm)	A few (diameter = 0.5 μm)	
Cell contacts	With other FLCs	With ALCs and fibroblasts	25 nm space contacts with adjacent LC
Microvilli			Accumulation in localized spaces
Surface	Numerous (finger-like protrusions)	A few, small protrusions	
Basal lamina	Present (of various thickness)	Absent	
Arranged	In clusters	Not exclusively in clusters	

LC, Leydig cells; ALCs, adult Leydig cells; ER, endoplasmic reticulum; FLCS, fetal Leydig cells; SER, smooth endoplasmic reticulum. (Reprinted from ref. *3*.)

contacts between perivascular FLCs and the endothelial cells of blood capillaries are often observed. These capillaries are fenestrated. Gap junctions and special desmosome-like cell contacts are observed between adjacent FLCs (Fig. 4). Intercellular bridges with continuous cytoplasm are present between the FLCs within a cluster during the early postnatal period (Fig. 4). Coated pits (CP) are observed rarely in these cells. The FLCs build the clusters for the first time on FD 16 and are surrounded by a basal lamina (basement membrane) and by a sheath of spindle-shaped fibroblasts at the outermost boundary. The basal lamina is nearly continuous during the fetal period and completely surrounds the FLCs cluster. Postnatally, however, the basal lamina becomes discontinuous and patchy. The basal lamina contains collagen type IV and laminin *(3,6,7)*.

Steroidogenic Function of FLCs

A histochemical reaction for 3β-HSD with an even, moderate intensity can be demonstrated in the cytoplasm of FLCs from FD 16 onward although, only a weak focal reaction can be observed on FD 14.5. The ontogenesis of 3β-HSD shows a peak on FD 19 and a sharp decrease immediately after birth *(5,8,9)*. The enzyme activity resides in the membranes of SER of the FLCs. Recent publications do not confirm the presence of a perimitochondrial or intramitochondrial enzyme reaction as previously reported *(10)*. The kinetic of testosterone synthesis in the FLCs coincides with the ontogenic behavior of 3β-HSD in the histochemical studies *(11–13)*. Also, the histochemical presence and ontogenic expression of 3α-HSD and 17β-HSD in the FLCs have been reported *(14–16)*. If rats are treated with an antiandrogen cyproterone acetate prenatally and early postnatally, the morphology and 3β-HSD histochemistry of FLCs remain unaltered *(17)*. In this case the FLCs clusters persist even longer (up to PND 32) than in the untreated controls. The oxidative activities of 11β-HSD types 1 and 2 are absent in the FLCs as shown by the histochemical studies (*15,18;* Fig. 5). The expression of LH receptors in the FLCs begins on FD 15.5 and increases from FD 18.5 onward. The steroidogenic factor SF-1 is present in the differentiated FLCs as well as in their precursors in the rat testis *(19)*. Androgen receptors are absent in the FLCs, but present in the peritubular cells from FD 16.5 onward *(20)*. Androgens from FLCs are essential

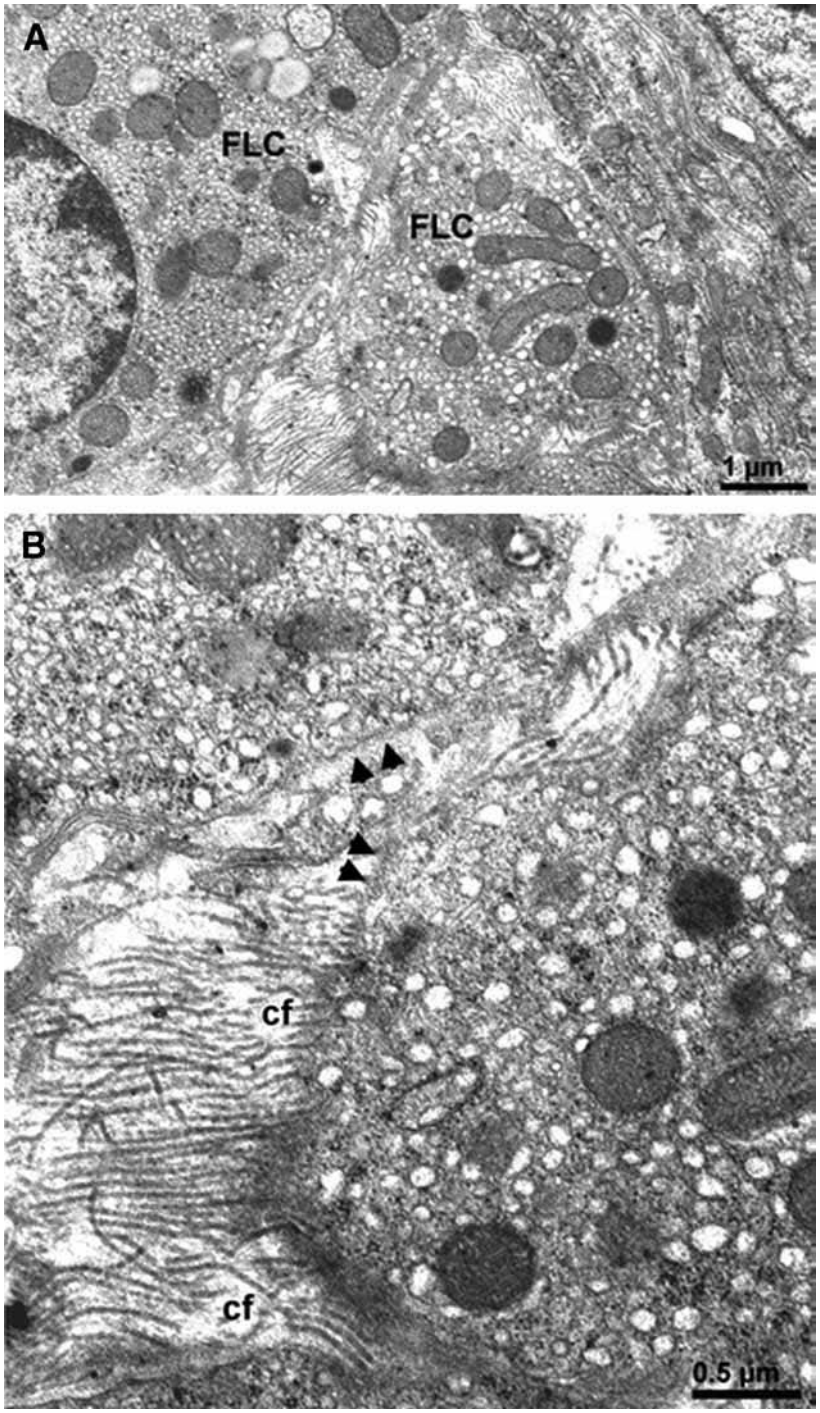


Fig. 3. (A) Ultrastructure of two FLCs on PND 10. The FLCs contain tubulo-vesicular mitochondria and SER. The middle portion of **(A)** is magnified in **(B)**. **(B)** The short arrows ◀ show the basal lamina around FLCs. The collagen fibers (cf) are attached to the basal lamina as well as to the plasma membrane of the FLCs.

for male gonadogenesis, formation of ALCs progenitors, differentiation and morphogenesis of male genital tract, masculinization, as well as sexual differentiation of the central nervous system.

The precursors of FLCs are mesenchymal fibroblasts and originate largely from the anterior lobe of the mesonephros and to a smaller extent from the gonadal ridge *(21–23)*. It seems that a common SF-1-positive

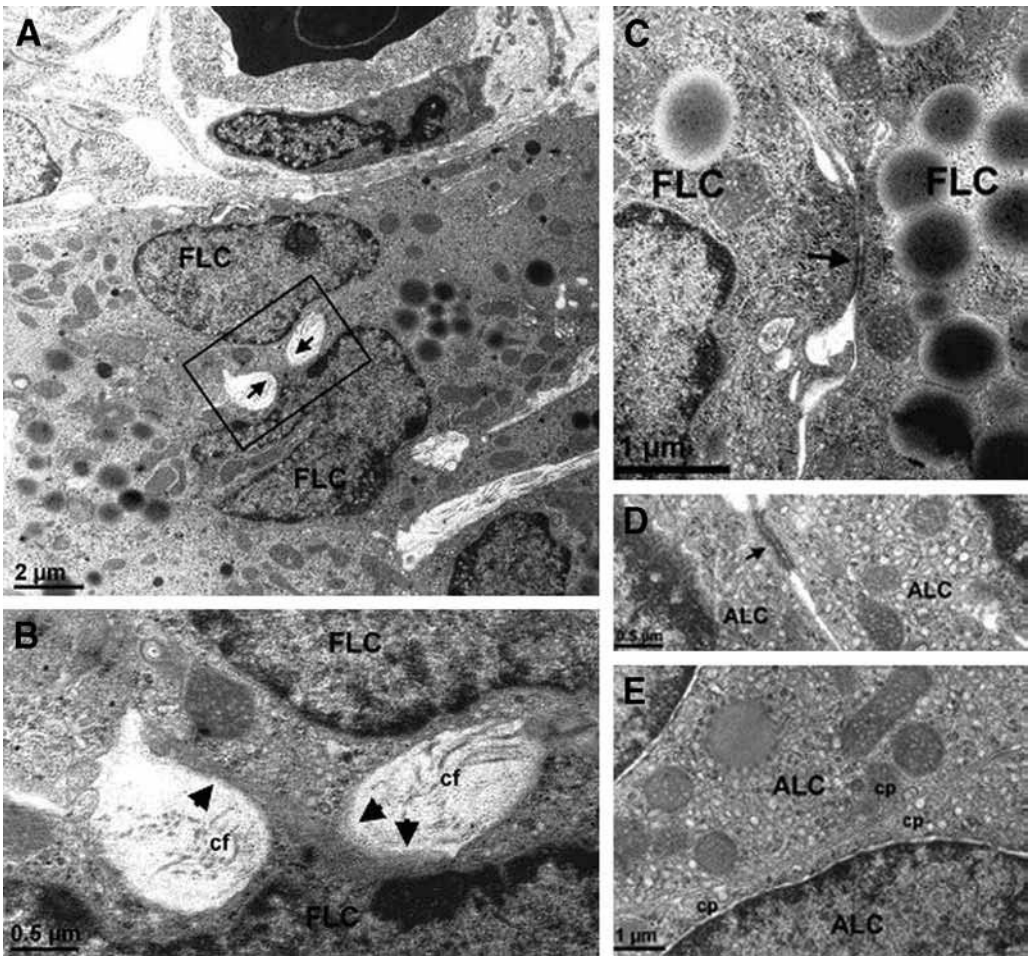


Fig. 4. Ultrastructure of cell contacts of FLCs and ALCs. **(A)** PND 1. An intercellular bridge between two adjacent FLCs in a cluster. **(B)** A magnification of a portion of Fig. 7A, showing the intercellular bridge. The cytoplasm between the two FLCs is continuous, the basal lamina (arrows)(◄) is present on both sides of the bridge. The intercellular space is filled with collage fibers (cf). **(C)** PND 1. A gap junction (arrow)(◄) between two adjacent fetal Leydig cells. **(D)** PND 30. A gap junction (arrow)(◄) between two immature ALC. **(E)** PND 30. Several coated pits (cp) at the cell membranes of two adjacent immature ALC.

steroidogenic population is shared and divided between the adrenal cortex and the fetal testis (ref. *24;* for details *see* Chapter 3). One aspect remains controversial: the ultimate fate of FLCs. They cannot be detected after PND 25 in the rat testis at light microscopic level. Do they involute or undergo apoptotic death? Or do they persist further? An interesting observation is the presence of 17β-HSD-10 in FLCs even after the time-point, when they begin to involute postnatally *(16)*. Caspase-3 and procollagen in the FLCs (Fig. 6A,B), and CF from the basal lamina surrounding the FLCs clusters (Fig. 3) have been observed. This suggests that the FLCs apoptosis is indeed followed by a process of hyalinization, which ultimately leads to the complete dissolution of FLCs. In contrast, some authors have reported the presence of FLCs till PND 90 *(25,26)*.

ADULT LEYDIG CELLS

Hardy et al. subdivided the differentiation of ALCs into three stages *(27),* which are as follows:

1. *Progenitor stage:* Leydig cells originate from mesenchymal-like fibroblasts (PND 13–28) and produce androsterone as the predominant androgen end product.
2. *Immature stage:* Leydig cells produce small amounts of testosterone from PND 35 on and metabolize most of the testosterone, the predominant androgen end product being 5α-androstane-3α, 17 β-diol.
3. *Mature stage:* Leydig cells actively produce testosterone as androgen end product and are fully functional in the sexually mature animal by PND 90 (refs. *28,29*; for details *see* Chapter 4).

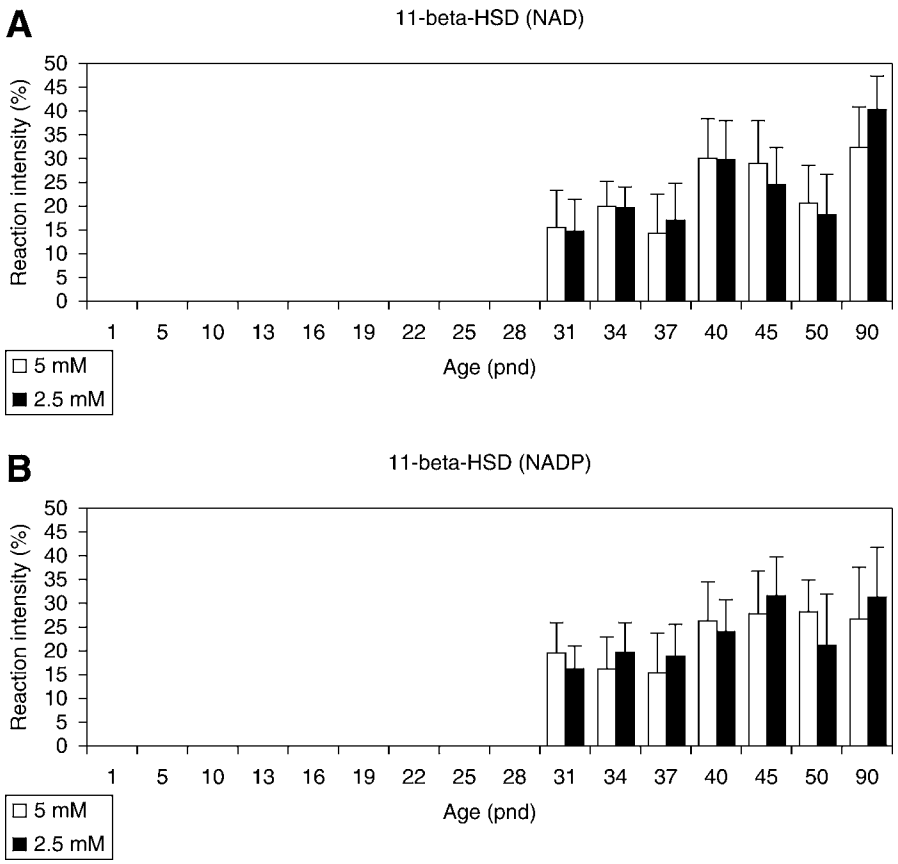


Fig. 5. Spectrophotometric measurement of the intensity of 11β-HSD histochemical reaction (substrate: corticosterone) in ALCs of rat with two different substrate concentrations (white column = 5 m*M*, black column = 2.5 m*M*). (Reproduced from ref. *15*.)

Histology and Ultrastructure of Progenitors of ALCs

The ALCs progenitors (PLCs)—also described as ALCs precursors by some authors *(3)*—originate from peritubular and perivascular fibroblasts in the rat testis (refs. *7,8,25*; Figs. 7A and 6C). The exact quantitative share of these two sources of PLCs is not known. However, the peritubular fibroblasts seem to be the major source for providing PLCs during ontogenesis *(7,8)*. During PND 10–13, the lamina propria (boundary tissue) of the seminiferous tubules consists of two layers of mesenchymal fibroblasts: the inner and the outer layer. The outer layer, directed toward interstitium provides progenitors for the ALCs, as identified by the presence of 3β-HSD and LH receptors. The formation of PLCs starts at the peritubular space between PND 10 and 13. It continues nearly 2–4 d. After 2–4 d at the perivascular space, where the mesenchymal fibroblasts around a blood capillary (BC) or blood vessel which are also called pericytes (PC)—begin with the expression of 3β-HSD *(10)*. These peritubular as well as perivascular progenitors are thin slender shaped cells with elongated nuclei (Fig. 6C). Until PND 9, these cells show morphological features typical for a fibroblast. However, from PND 10–13 the cells in the outer layer of the peritubular cells show the following additional features: SER, tubulo-vesicular mitochondria, and lipid droplets signs typical for a steroid-synthesizing cell (Fig. 7A). The same is true for the PLCs at the perivascular space. These progenitors show the highest labeling index and a duration of DNA synthesis (S phase) of 10 h from PND 9 to 23, as revealed by the autoradiographic double labeling experiments with 3H-thymidine and 14C-thymidine. The absolute number of fibroblasts shows a peak on PND 13 and then gradually decreases *(7)*. The PLCs contain LH receptors, androgen receptors, 3β-HSD, 3α-HSD, and 17β-HSD but not 11β-HSD *(15,18,30)*. The complete destruction of ALCs after EDS treatment is followed by a regeneration of newly formed ALCs; thereby pericytes and vascular smooth muscle cells seem to provide progenitors of ALCs *(31)*.

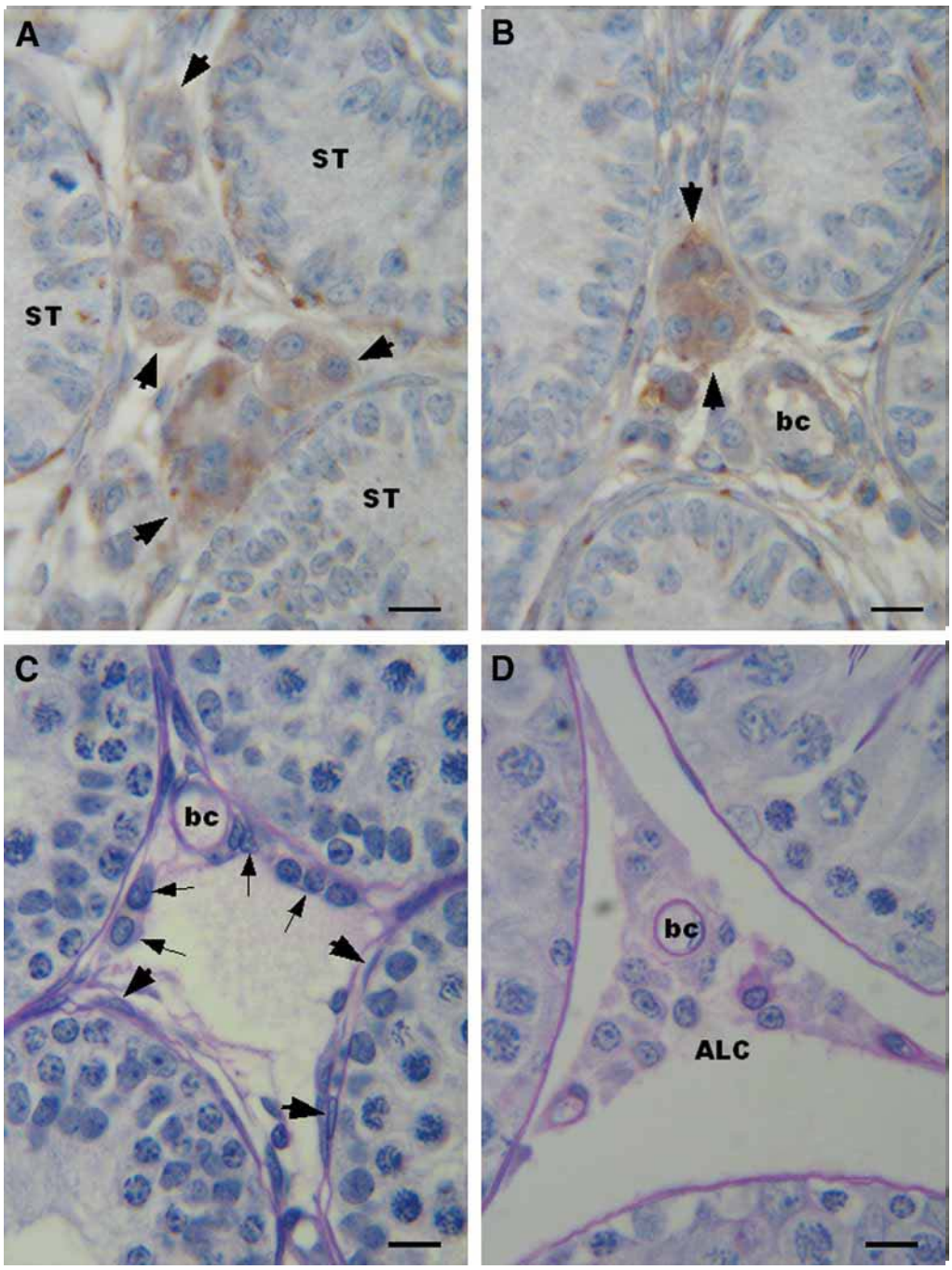


Fig. 6. Bar for **(A–D)**: 10 μm. (**A**,**B**): Immunohistochemical reaction of procollagen (brown color) in FLCs on PND 5. (**A**) The arrows ◄ show two FLCs clusters. Only two FLCs show immune reaction in the upper cluster, other FLCs are negative. In the lower cluster all FLCs are positive. (**B**) Two arrows ◄ point to a FLCs cluster, the FLCs are homogeneously positive for procollagen. (**C**,**D**): PAS with hematoxylin staining. (**C**) PND 15. Round to oval-shaped progenitors of ALCs with peritubular as well as perivascular location (arrows)(◄). Three short arrows ← point toward thin elongated myofibroblasts lying in the inner layer of the lamina propria (peritubular cells), and blood capillary (bc). (**D**) ALCs on PND 60. The ALCs are arranged around a bc. (Please *see* color version of this figure on color insert following p. 180.)

Histology and Ultrastructure of Immature and Mature ALCs

The immature ALCs (ILCs) are unlike PLCs oval or nearly round with a round nucleus and acidophilic (eosinophilic) cytoplasm, and are present during PND 25–35 in the rat. Unlike FLCs, the immature as well as mature ALCs are not arranged exclusively in clusters. The peritubular as well as perivascular

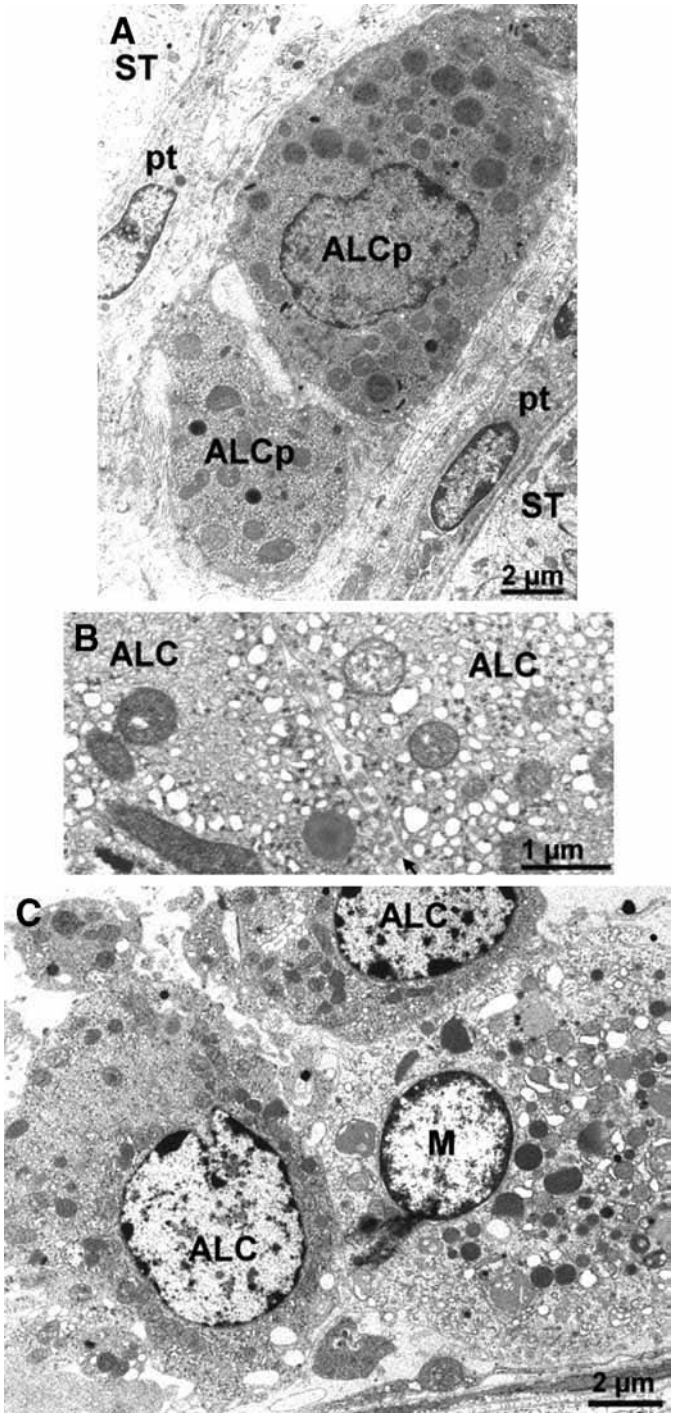


Fig. 7. (A) Ultrastructure of progenitors of ALCs (ALCp) on PND 15. (**A**) peritubular myofibroblast (pt) in the inner layer of the lamina propria around a seminiferous tubule (ST.) **(B)** Immature ALC with tubular mitochondria and SER on PND 50. Note the abundant secretory vesicles (*arrow*) between the two adjacent ALCs (**C**) Two mature ALC with a macrophage (M) on PND 100.

PLCs grow in size changing their shape from slender to oval or round. In the course of transition to ILCs, the peritubular PLCs move away from the seminiferous tubule (ST) toward the interstitium preferably searching a place in close association with blood capillaries. The cellular and molecular mechanism that initiate and regulate the movement of the peritubular PLCs toward the interstitium are unknown. On PND 30, most of the ILCs are evenly distributed in the interstitial space with a BC in the near vicinity. Unlike FLCs, ALCs are not surrounded by a basal lamina and are not always covered by a sheath of fibroblasts.

The nuclei of immature as well as of mature ALCs are large and round with condensed euchromatin and with one or two nucleoli (Figs. 6D, 7B,C). The cytoplasm contains large amounts of SER, a high number of tubulovesicular mitochondria, a well-differentiated Golgi apparatus, and a few small lipid droplets with an average diameter of 0.5 µm (refs. *3,7,32–34*; Table 1). The presence of microvilli is concentrated only focally. Membrane protrusions are observed only occasionally; the conspicuous interdigitating long membrane protrusions, present in FLCs, are absent here. Gap junctions and coated pits are observed at the surface of ALCs membranes. Gap junctions are present in immature as well as in mature ALCs; a structural diversity as well as an increased formation of gap junctions (Fig. 4) with the maturity of the ALCs is observed. In general, the ALCs tend to form more gap junctions than the FLCs *(35)*. Coated pits are observed initially on PND 30. From PND 50 onward, macrophages and ALCs are attached very closely to each other, when the cell processes of ALCs protrude into the CP of the macrophage *(36)*. The immature ALCs exhibit more pinocytotic vesicles in the extracellular space between the adjacent ALCs than do in mature ALCs; this probably suggests a high secretory and transport activity in immature ALCs.

The aforementioned cell contacts between adjacent ALCs, as well as between ALCs and other interstitial cells probably constitute the morphological substrate for intercellular communication. The cell contacts between Leydig cells and closely associated vascular endothelial cells, and several endocrine studies suggest a close paracrine interdependence between ALCs and the vascular endothelial cells. Ghinea et al. *(37)* have described a receptor-mediated transendothelial transport of LH in rat testis. They found the LH/hCG receptors not only in Leydig cells but also in endothelial cells. These receptors are involved in hormone transcytosis through endothelial cells *(38)*. In 12% of interstitial field, on PND 15, the reaction product of 3β-HSD is found not only in ALCs progenitors and FLCs, but also focally on SER membranes of the vascular endothelial cells in the rat testicular interstitium *(10)*. Leydig cells are able to produce vascular endothelial growth factor, and this process, mediated by cAMP-dependent protein kinase A, is under gonadotropic control *(39)*. The cytoplasmic digitations between Leydig cells and macrophages, and several cell contacts between these two cell types show a close paracrine relationship. The growth factors, secreted by testicular macrophages, are essential for differentiation and development of newly formed ALCs, and play a modulatory role for steroidogenesis in the Leydig cells *(40,41)*.

Steroidogenic Function of ALCs

Immature as well as mature ALCs express LH receptors, 3β-HSD, 3α-HSD, 17β-HSD, from PND 30 onwards also 11β-HSD (refs. *2,8,9,15,27,30,42–44*; for details *see* Chapter 10). The 3β-HSD enzyme resides on SER membranes of immature and mature ALCs *(10)*. The percentage of the area of 3β-HSD-positive Leydig cells relative to a cross-section of the testis increases from 0.3 on PND 11 to 1 on PND 13. Thereafter it decreases to 0.3 up to PND 23 followed by an increase up to 5 on PND 25. On PND 29, it decreases once again to the level of 0.7. If rats are treated with the antiandrogen cyproterone acetate prenatally and postnatally. The number of 3β-HSD positive PLCs decreases significantly. This treatment delays the transformation of PLCs, but does not block it completely *(17)*. Schäfers et al. *(15)* described ontogenic changes in histochemical distribution and intensity of the oxidative reaction of 17β-HSD and 11β-HSD in Leydig cells from PND 1–90. For 17β-HSD (substrate: 5-androstene-3β,17β-diol) a peak was observed in the ALCs on PND 19 and 37. Leydig cells synthesize and maintain a high level of 17β-HSD type 4 *(45)*. The histochemical oxidative reaction for 11β-HSD (substrate: corticosterone) is present from PND 31 onwards, first in few ALCs and later from PND 40 onwards in all ALCs homogeneously; the intensity of the reaction is nearly identical with NAD or NADP as cofactor (Fig. 5). The enzyme 11β-HSD possesses a unique position among all HSD enzymes in Leydig cells. This is the only HSD enzyme, which could not be traced histochemically in the FLCs, whereas the ILCs start expressing this enzyme from PND 31 onward. Its expression coincides temporally with the first formation of elongated spermatids in the rat testis on PND 35 *(43)*. The period PND 35–40 seems to be a turning point in the steroidogenic capacity of ALCs. Ge et al. *(46)* have confirmed the presence of 11β-HSD type 1 (cofactor NADP) and of 11β-HSD type 2 (cofactor NAD) in Leydig cells. The loss of androgen receptor-mediated androgen action has major consequences for the development, number and function of ALCs *(47)*. The main intratesticular function of testosterone from ALCs is to support meiotic and postmeiotic germ cell development and Sertoli cell proliferation *(48)*.

Russell *(33)* published an excellent comprehensive review on comparative aspects of structure and ultrastructure of Leydig cells in various mammalian

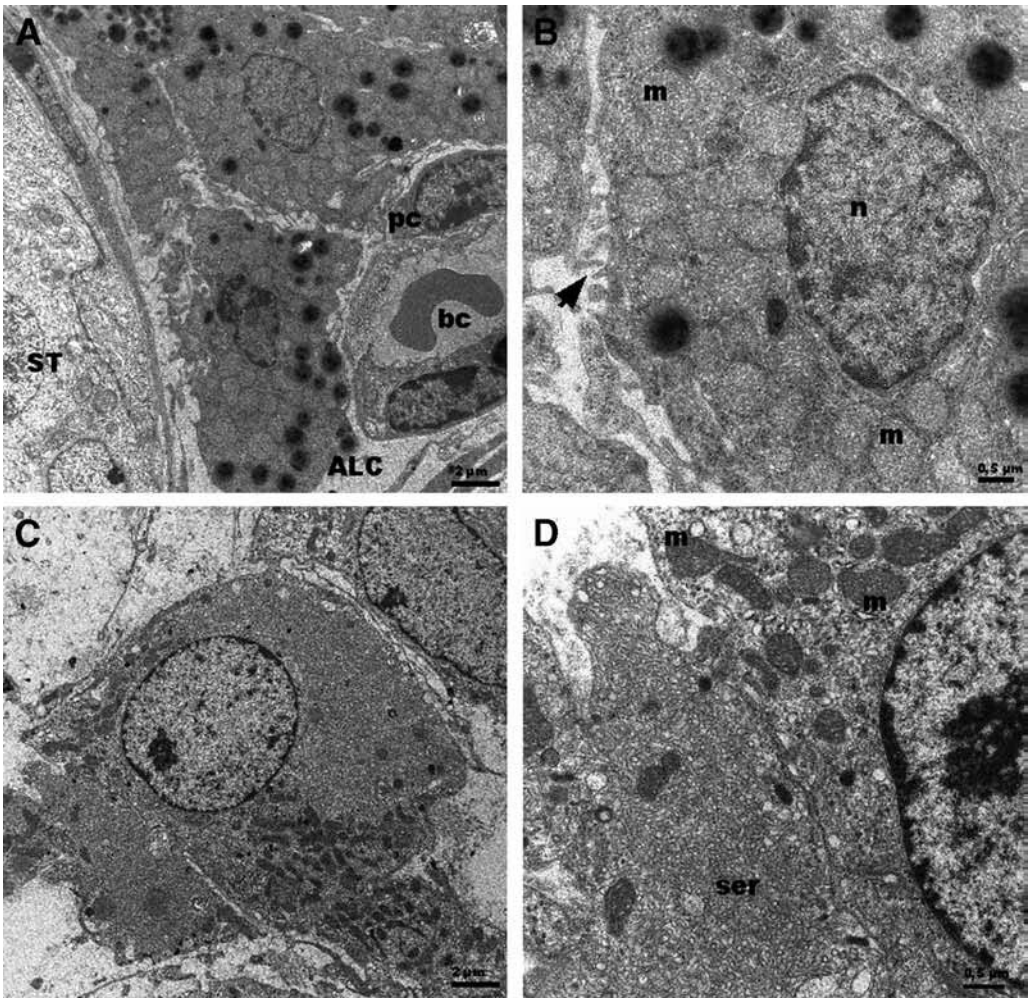


Fig. 8. Ultrastructure of ALCs of a mouse (**A**,**B**), and a marmoset new world monkey (**C**,**D**). (**A**) ALCs, seminiferous tubule (ST), blood capillary (bc), and pencyte (pc). The upper middle portion of (**A**) is magnified in (B). (**B**) The vesicular type of mitochondria (m) is dominating, nucleus (n). Secretory vesicles (arrow)(◄) at the cell membrane. (**C**) Marmoset ALCs. The mitochondria are located mainly in the lower part and the SER in the upper part of the cell. The left portion of the (**C**) is magnified in (**D**). (**D**) Smooth endoplasmic reticulum (ser), and mitochondria (m).

species. These studies revealed distinct patterns of organization of interstitial structures and varying abundance of Leydig cells. The ALCs in the mouse and rat *(49,50)* are organized similarly, i.e., large spaces occupied by peritubular lymphatic sinusoid with a relatively small volume of Leydig cells and little connective tissue stroma. Figure 2E–G show 3β-HSD in ALCs of mouse, pig, and bull testis. The Leydig cells in adult pigs are organized and arranged in two different topographic patterns: (1) "intertubular" Leydig cells along the testicular septae around a group of seminiferous tubules and (2) "peritubular" Leydig cells around a single ST (Fig. 2F). The ultrastructure of ALCs of mouse and of marmoset new world monkey (*Callithrix jacchus*) is shown in Fig. 8.

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3 Fetal Leydig Cells

Origin, Regulation, and Functions

Humphrey Hung-Chang Yao, PhD and Ivraym Barsoum, MD/MS

SUMMARY

The discovery of adult Leydig cells by Franz Leydig in 1850 led to extensive research into the functions and regulation of this testis-specific cell type *(1)*. In the early 1900s, Whitehead presented the first extensive analysis of fetal Leydig cells (FLCs) in pig embryos, staging the idea that a distinct Leydig cell population exists in fetal testes *(2,3)*. Since then, it is widely accepted that at least two populations of Leydig cells—fetal and adult types—exist at different developmental stages in most mammalian testes. Despite their differences in morphological and biochemical properties, fetal and adult Leydig cells share a common function, i.e., production of androgens. FLCs have been characterized in a wide range of mammalian species including human, monkey, pig, rabbit, guinea pig, hamster, rat, and mouse. This chapter focuses on the morphological characteristics, origin, specification, and functions of FLCs in rats and mice, species in which most physiological and genetic experiments are conducted.

Key Words: Adrenal–gonadal primordium; androgens; fetal Leydig cells (FLCs); gonad; mesonephros; Sertoli cells (SC); testis; testis descent; Wolffian duct.

CHARACTERISTICS OF FLCs

Anatomical Location and Morphology

In most mammalian species such as rodents and human, FLCs start to appear in the mesenchyme of the developing testis immediately following the formation of testis cords (embryonic day 12.5 or E12.5 in mice, E14.5 in rats, and 7–8 wk of pregnancy in human *see* Chapter 5). As FLCs differentiate outside of the testis cords, they transform from spindle to oval in shape and acquire typical steroidogenic cell characteristics, such as abundant smooth endoplasmic reticulum, mitochondria of tubulovesicular type and lipid droplets *(4–6)*. These ultrastructural characteristics of FLCs are very similar to those in adult Leydig cells with the exception that FLCs have a less-developed Golgi apparatus, more and larger lipid droplets, and numerous protrusions on the cell membrane *(7)*. Furthermore, unlike adult Leydig cells, FLCs form clusters and are surrounded by a basal lamina that contain extracellular matrices such as collagens and laminin *(4,8)*. The basal lamina becomes discontinuous and the clustering of FLCs starts to break up after birth *(4)*. Gap junctions are prominent among the clustered FLCs and intimate cellular connections are often observed between FLCs and endothelial cells of vasculature *(7)*.

Developmental Course

The number of FLCs increases dramatically once their differentiation begins despite the fact that they are mitotically inactive (low incorporation of [^{3}H]-thymidine) *(9)*. This observation suggests that the transformation of precursor cells in the interstitium and/or other sources (*see* "Cellular Origin" section) are primarily responsible for increases in FLC numbers. In rats, FLC numbers increase from 2.5×10^4 per testis at E17 to about 1×10^5 cells per testis at E21 or immediately before birth *(10)*. The volume of FLCs per testis corresponds to changes in cell numbers and increases from 4% at E16.5 to about 9% at E18.5. However, the volume of FLCs decreases after E18.5 and continues to

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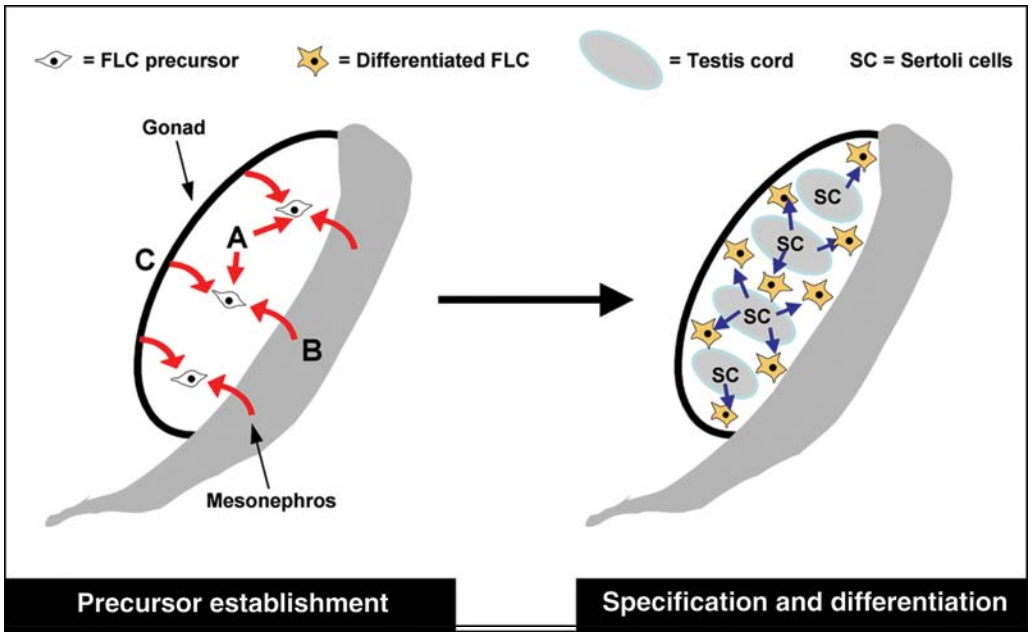


Fig. 1. Origin, specification, and differentiation of FLC. FLC has been shown to originate from at least three possible sources. **(A)** Gonadal mesenchyme that shares a common primordium as the adrenal cortex. **(B)** Cells that migrate from the mesonephros. **(C)** The coelomic epithelium that encapsulates the gonad. Once the precursor population of FLC is established, Sertoli cells (SC) in the testis cords produce morphogens including DHH, PDGF, and other yet-to-be-identified molecules to specify the FLC lineage and to induce their differentiation. (Please *see* color version of this figure on color insert following p. 180.)

decline after birth and during the first and second weeks after birth *(11–14)*.

The decline of FLCs after birth has led some researchers to question their developmental fate in adults. At least in rats, it was suggested that FLCs could either undergo degeneration *(5,11,12)*, transform into adult FLCs *(15)*, or persist in adult testis along with the adult Leydig cell population *(10,16)*. The fact that fetal and adult Leydig cells share similar morphological characteristics, steroidogenic activity and regulation, and expression of various markers suggests that they could derive from the same origin and represent different stages of development. However, the ability of adult Leydig cells to repopulate the testis from vasculature progenitors after ethane dimethanesulphonate treatment suggests otherwise; adult Leydig cells could be a steroidogenic population distinct from FLCs *(17)*. More experiments such as identification of markers specific for each Leydig cell population and lineage tracing experiments are critical to settle this issue.

ORIGIN AND SPECIFICATION OF FLCs

Cellular Origin

The origin(s) of FLC precursors has been the center of debate for years. FLCs could originate from four possible sources: adrenal–gonadal primordium (mesoderm), neural crest (neuroectoderm), mesonephros (mesoderm), and coelomic epithelium (endoderm) (Fig. 1).

Abilities of the adrenal gland (specifically the cortex) and gonad to produce androgens and other steroid derivatives have prompted the idea that FLCs share a common origin with cells in adrenal cortex *(2,18–22)*. Indeed, adrenal cortex and gonads are both derived from the common adrenal–gonadal primordium before they separate into two identities *(19,23)*. Steroidogenic factor 1 (*Sf1*), a transcription factor essential for the specification of steroid-producing cells as well as for the expression of steroidogenic enzymes, is expressed in the coelomic epithelium and mesenchyme of the adrenal–gonadal primordium *(24)*. Expression of *Sf1* continues in the adrenal cortex and gonad when their common primordium separates into two distinct cell populations. *Sf1*-positive cells in gonadal primordium eventually differentiate into Sertoli cells (SC), Leydig cells, and other somatic cells. When *Sf1* is inactivated, both adrenal glands and gonads undergo degeneration *(25–27)*. This observation further supports the idea that the *Sf1*-positive cell population in the adrenal–gonadal primordium is one source of gonadal somatic cells, possibly the FLCs.

Fetal and adult Leydig cells are known to express neural cell adhesion molecule (NCAM), neurofilament protein 200, and microtubular-associated protein *(17,28–36)*. The fact that FLCs exhibit these neural characteristics and markers have led to the hypothesis that Leydig cell precursors are derived from the neural crest. However, Brennan et al. *(37)* examined two neural crest lineage tracing mouse lines and found no evidence of neural crest contribution to FLC population. This finding suggests that FLCs probably do not originate from the neural crest and that their neural properties are acquired during differentiation in the testis.

The mesonephros, a tissue that contains the precursors of the reproductive tracts (Wolffian duct and Müllerian duct), is another source of FLC precursors. The gonadal primordium develops on the dorsal-medial part of the mesonephros along the anterior–posterior axis and establishes a close anatomical connection with the mesonephros. The mesonephros connects to the gonadal primordium through an elaborate ductal system, which is thought to provide founder cells in gonads *(38)*. The contribution of the mesonephros to gonadal cell populations was examined by a gonad/mesonephros recombinant culture: normal gonads were grafted on top of the mesonephros carrying reporter genes such as green fluorescent protein (GFP) or LacZ. GFP- or LacZ-positive cells from the mesonephros migrated into the gonad and some of them became steroidogenic *(39–43)*. Interestingly, when an E11.5 XY mouse gonad was cultured without the mesonephros attached, some Leydig cells still appeared, suggesting that, if precursors for Leydig cells migrate from the mesonephros, they must do so before E11.5 *(44)*.

Another potential source of FLC precursors is the coelomic epithelium that encapsulates the gonad. The coelomic epithelium is positive for *Sf1* and proliferates actively during testis morphogenesis. Progenies of the proliferating coelomic epithelium delaminate from the epithelium, move into the gonad, and differentiate into SC and interstitial cells, possibly FLCs *(45,46)*. Taken together, these evidence suggest that precursors for FLCs derive from multiple sources and acquire Leydig cell characteristics when they are exposed to the testicular environment. It remains to be determined whether these different sources provide a homogenous or mixed population of progenitor cells that eventually, develop into a single Leydig cell linage.

Molecular Regulation of FLC Differentiation

FLCs appear only in embryonic testes suggesting that their differentiation is regulated by either the sex chromosome composition or a testis-specific mechanism. Burgoyne and colleagues demonstrated, by generating chimeric mouse embryos with XX and XY cells, that an equal portion of XX and XY cells contribute to FLC population *(47)*. A bias toward the XY genotype only occurs in SC in this XX<–>XY chimera. Apparently, presence of the Y chromosome does not directly dictate the appearance of FLCs. Indeed, the testis-determining gene *Sry* (sex-determining region of the Y chromosome) is expressed only in SC and not in Leydig cells *(48–52)*. These observations support the idea that FLC differentiation is indirectly regulated by *Sry*, through the production of morphogens by the *Sry*-expressing SC (Figs. 1 and 2).

Desert hedgehog (Dhh) is the first Sertoli cell-derived morphogen that is found to be essential for Leydig cell differentiation *(53–55)*. In XY mouse embryos, *Dhh* is expressed in SC, whereas its receptor *Ptch1* is expressed in interstitial cells outside of testis cords where precursors of FLCs reside *(53,56)*. Mouse embryos carrying a null mutation of *Dhh* have a severely reduced Leydig cell population and insufficient androgen production, leading to pseudohermaphroditic phenotypes such as undescented testes, atrophic male internal accessory organs, and female external genitalia *(55–57)*. Mutations in the human *DHH* gene lead to the pseudohermaphroditic phenotypes as well as gonadal dysgenesis, similar to the *Dhh*-null mice *(58,59)*. DHH appears to act as a paracrine factor to trigger differentiation of Leydig cell precursors, particularly the expression of *Sf1* and P450 side-chain cleavage enzyme (*Scc*). However, once Leydig cells differentiate, they no longer require the presence of DHH *(56)*. In addition to *Dhh* mutants, a decrease in Leydig cell numbers is also observed in XY embryos carrying a null mutation of platelet-derived growth factor receptor (PDGFR)-α *(37)*. PDGFR-α is required for specification and/or expansion of the Leydig cell population *(37)*. The DHH and PDGF signaling pathways appear to operate in parallel to regulate Leydig cell differentiation based on findings that components of the PDGFR-α pathway remains unchanged in *Dhh* null mutant (and vice versa). Defects in Leydig cell development are reported in mice carrying a null mutation of insulin-like growth factor 1 gene (*Igf1*) as well *(60)*. *Ifg1* is expressed in fetal testes and is able to increase the number and steroidogenic capacity of dispersed fetal testicular cells in culture *(61)*. These observations together demonstrate that Leydig cell differentiation is regulated by a network of signaling pathways originating from SC and/or other somatic cells. It remains unknown how these different signaling molecules interact to facilitate Leydig cell differentiation.

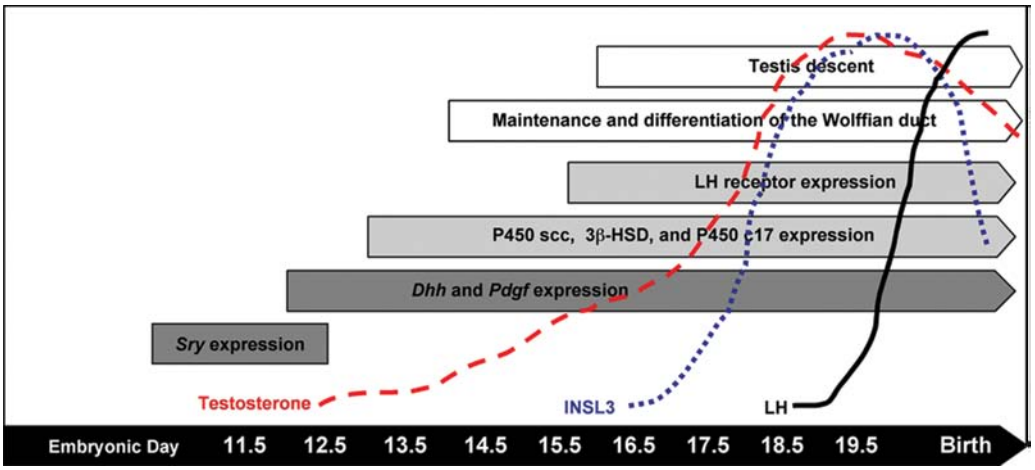


Fig. 2. Developmental landmarks in fetal testes of rats and mice from E11.5 to birth. *Sry*, which is expressed between E10.5 and E12.5, induces differentiation of Sertoli cells (SC). Immediately following *Sry* expression, SC express *Dhh* and *Pdgf*, two morphogens that regulate FLC development. At E13.5, FLCs begin to express steroidogenic enzymes, including P450scc, 3β-(HSD) hydroxysteroid dehydrogenase, and P450c17, and produce androgens. Androgen production (in rats) reaches its peak right before birth and declines after birth. Production of INSL3, another molecule produced in FLCs, starts to appear at E15.5 and declines immediately before birth. Receptors for LH do not appear on FLCs until E15.5 or later and LH only becomes detectable in the serum 24–48 h before birth, indicating that the initial differentiation of FLCs is LH-independent. The broken, dotted, and solid lines represent changes of testosterone, INSL3, and LH, respectively. The levels of individual molecules are arbitrary and cannot be used for comparison between molecules. The white, light gray, and dark gray horizontal bars represent developmental landmarks of the reproductive tract, FLCs, and SCs, respectively. (Please *see* color version of this figure on color insert following p. 180.)

Ectopic *Scc*-positive cells were initially reported in the female gonads carrying a null mutation of *Wnt4* (a morphogen expressed exclusively in female gonads at the time of sexual differentiation) *(62)*. It was therefore postulated that Leydig cell differentiation in the XY gonad is a default event, which is suppressed in the XX gonad by *Wnt4*. However, further analysis revealed that these steroid-producing cells in *Wnt4*-null gonads not only express steroidogenic enzymes specific for adrenal cortex but also appear in gonads in both sexes (not a female specific phenotype) *(63)*. Therefore, instead of suppressing FLC differentiation, *Wnt4* appears to be important for proper allocation of steroidogenic cells when adrenal–gonadal primordium separates into two organs.

In addition to paracrine factors, various transcription factors are also involved in differentiation of FLCs. Null mutation of *Pod1* (capsulin/epicardin/Tcf21), a helix-loop-helix transcription factor expressed in the interstitium of gonads, causes a dramatic increase of *Scc*-expressing cells in both XY and XX gonads *(64)*. However, FLCs in *Pod1*-null gonads produce no or a low amount of androgens as both mutant XX and XY embryos fail to virilize. *Pod1* could act to prevent premature differentiation of FLCs and at the same time, control proper steroidogenesis once FLCs arise. Defects in FLC differentiation are also reported in mice and humans carrying mutations in X-linked aristaless-related homeobox gene (*Arx*) *(65)*. Notably, *Arx* is not present in FLCs in mice, suggesting that *Arx* may indirectly control FLC development. Estrogen receptor (ER)α is also expressed in FLCs *(66)*. When ERα is inactivated, FLCs undergo hypertrophy and produce a higher amount of testosterone *(67)*. This observation indicates that endogenous estrogens inhibit steroidogenesis of FLCs through ERα. Signaling pathways induced by retinoic acids are also shown to inhibit steroidogenesis of FLCs *(68–72)*. These experiments have yielded a tremendous amount of information on FLC differentiation but further studies are needed to understand how these transcription factors interact with each other and what is their relationship with paracrine factors.

Unlike adult Leydig cells, whose development is controlled by luteinizing hormone (LH), differentiation of FLCs is LH-independent in rodents based on the following observations (Fig. 2) *(73–75)*. First, FLCs differentiation occurs 48–72 h before they start to express LH receptor *(76,77)*. Furthermore, LH is detected in the plasma at least 72 h after the appearance of FLCs *(78,79)* and remains low until before birth *(13)*. Third, differentiation of FLCs proceeds normally when XY gonads are separated from the embryos and are cultured in vitro *(8,77,80,81)*. Finally, in hypogonadal mice (gonadotropin deficiency) *(75)*, LH β-subunit knockout mice *(82)*, and LH receptor knockout mice *(83)*, FLCs differentiate normally. Interestingly, decapitated rat embryos produce less testosterone than control

embryos *(13,84,85)*. LH replacement was able to restore testosterone production in these decapitated embryos, suggesting that LH becomes essential for FLCs to produce testosterone later in fetal life *(86)*. This evidence clearly demonstrates that initial development and maintenance of FLCs do not require LH. However, steroid synthesis by fetal Leydig cells gradually becomes LH-dependent immediately before birth.

FUNCTIONS OF FLCs

FLCs produce an array of molecules ranging from steroids to cytokines. Among these molecules, functions of androgens and insulin-like factor 3 (INSL3 or relaxin-like factor) are the most critical and well-defined. Androgens produced by FLCs regulate differentiation of the Wolffian duct and external genitalia (*see* "Development of the Wolffian Duct and Male External Genitalia" section) and both androgens and INSL3 are involved in testis descent (*see* "Testis Descent" section).

Development of the Wolffian Duct and Male External Genitalia

In the 1940s, Alfred Jost first identified the importance of testis-derived androgens on the development of the Wolffian duct. Using rabbit embryos, he demonstrated that castration led to degeneration of the Wolffian duct and that this degeneration could be rescued by testosterone replacement *(87,88)*. The source of testosterone in the testis was not known until late 1950s when Wattenberg identified Leydig cells as the only cell type in the testis expressing the steroidogenic enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) *(89)*. FLCs contain intracellular organelles known to be unique for steroidogenic cells (*see* "Characteristics of FLCs" section) and steroidogenic enzymes for androgen synthesis such as 3β- and 17β- hydroxysteroid dehydrogenase (3β-HSD and 17β-HSD) *(74,77,90–95)*, resembling their adult counterparts. But unlike the adult Leydig cells, FLCs do not contain corticosteroid 11β-dehydrogenase, indicating that FLCs are unable to metabolize cortisol to cortisone *(96)*. The necessity of androgens on Wolffian duct development is further confirmed by genetic experiments and clinical cases. Inactivation of androgen receptor in mice *(97–99)* and humans (androgen insensitivity) *(101)*, results in a loss of the Wolffian duct in genetic XY individuals. In contrast, when XX individuals are exposed to excessive androgens during fetal development such as the case of adrenal hyperplasia, the Wolffian duct remains and develops into the male reproductive tract.

In the genital tubercle, the precursor of the external genitalia, testosterone produced by FLCs is converted to 5α-dihydrotestosterone (DHT) by the enzyme 5α-reductase *(102)*. DHT, instead of testosterone, is the main factor that sculpts the genital tubercle into male external genitalia, including the penis and scrotum.

Testis Descent

FLCs also produce INSL3, which along with androgens, play essential roles in testis descent (Fig. 2). Descent of testes into the scrotum in mammals is critical because proper spermatogenesis requires a lower temperature. Testis descent is a two-stage event including the transabdominal migration (INSL3-dependent) and inguino-scrotal descent (testosterone-dependent). Before the production of INSL3 and testosterone, embryonic gonads are loosely held by the dorsal ligament (cranial suspensory ligament or CSL) and ventral ligament (later develops into gubernacula). In the male, testosterone causes involution of the CSL, whereas INSL3 induces massive growth of the gubernaculum through LGR8 receptor during the transabdominal migration *(104,105)*. The thickening gubernaculum retain the testes close to the inguinal region, whereas the rest of the abdominal contents grow dorsally. In the second migration step, the inguinal canal and scrotum are formed under the influence of testosterone, facilitating the passage of testes into the scrotum *(106)*. In the female embryo, in which FLCs are absent (therefore no testosterone and INSL3 are produced), the CSL continues to develop, whereas the gubernaculum involutes, maintaining the ovary close to the kidney *(107)*. Inactivation of either testosterone or INSL3/LGR8 pathway leads to cryptorchidism *(108–110)*. Furthermore, when the INSL3/LGR8 pathway is ectopically activated in the female embryo, transabdominal migration of the ovary takes place, but the inguino-scrotal descent, does not occur *(107)*. These observations indicate that a complete descent of testes requires both testosterone and INSL3 from FLCs.

PERSPECTIVE

Although the physiological and evolutionary significance of having two distinct Leydig cell populations during male development remains an enigma, this uncertainty has not diminished the efforts to search for the cellular origin, molecular mechanisms, and biological functions of FLCs, the cell type that establish the phenotypic maleness of the individual. The everlasting debate on whether fetal and adult Leydig cells belong to the

same lineage, or they are simply unrelated, could turn out to be more philosophical than scientific. Nevertheless, an understanding of how FLCs behave and differentiate will not only provide clues on congenital defects in male sexual development but also identify potential applications to control androgen production and fertility.

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4 Regulation of Leydig Cells During Pubertal Development

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SUMMARY

Adult Leydig cells are terminally differentiated cells with an organelle structure adapted to serve in steroidogenesis. Adult Leydig cells are formed during pubertal development from a precursor cell population, through a series of intermediate stages beginning ultimately with a stem cell designated as a stem Leydig cell. The stem Leydig cells are distributed in the interstitial space and might be centrally located or adjacent to peritubular cells that sit immediately atop the basal lamina of the seminiferous tubule, or to blood vessels. The ultimate origin of stem Leydig cells remains a topic of active research, with most investigators favoring mesenchymal cells derived from the primitive kidney (mesonephros), but others supporting sources including the neural crest and coelomic epithelium, which later give rise to the tunica (testis capsule). Although it is firmly established that luteinizing hormone (LH) is the chief tropic stimulus of Leydig cell steroidogenesis, the process by which stem Leydig cells acquire the ability to respond to hormone stimulation is largely unknown. Growth factors produced locally by Sertoli cells, including Desert Hedgehog, platelet-derived growth factor, leukemia inhibitory factor, Kit ligand and insulin-like growth factor-1, may act sequentially or together to stimulate the transition from stem to later stage Leydig cell before LH sensitivity is acquired. Androgen, potentially secreted by fetal Leydig cells may be essential for initial development of adult Leydig cells. LH signaling is necessary to amplify cell numbers further and induce the differentiation of later stage Leydig cell intermediates. Puberty concludes with the creation, in the testis of the adult rat, of a population of about 25 million Leydig cells that produce testosterone.

Key Words: Desert Hedgehog; mesenchymal cell; neural crest; PDGF; progenitor Leydig cell; stem Leydig cell; steroidogenesis.

INTRODUCTION

Leydig cells synthesize and secrete the steroid hormone testosterone, and are the primary source of this androgenic hormone in the body. Testosterone, referred to as the male hormone, stimulates male sexual differentiation before birth, and fertility and male secondary sexual characteristics after birth. Receptors for testosterone (androgen receptors) are not confined to the reproductive system and are widely distributed in other tissues, including skeletal muscle *(1)* thymus *(2)*, and brain *(3)*. Thus, the differentiation of Leydig cells in the testes is of general importance in the development of the male body plan. For reasons that remain unclear, there are discrete phases of testosterone secretion during the life cycle: two in the rodent (fetal and adult) and three in the human (fetal, neonatal, and adult), which are products of separate generations of Leydig cells.

The first generation forms during embryogenesis and its members are accordingly designated fetal Leydig cells. The fetal Leydig cells differentiate from stromal cells between the nascent testis cords, starting on day 12 of gestation in rats. Shortly after the testis differentiates from the indifferent gonad, the fetal Leydig stem cells undergo lineage specific commitment and differentiate into mature fetal Leydig cells that are fully competent in steroidogenesis *(4)*. They reach their peak of steroidogenic activity just before birth on day 19 of gestation *(5)* and the testosterone secreted is critical for development of the penis and sex accessory glands *(4)*. Fetal Leydig cells also play a role in the scrotal descent of the testis, by synthesizing androgen and insulin-like growth factor-3 (INSL-3, also known as relaxin-like factor *[6]*). A receptor for INSL-3, LGR-8, is present

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in the gubernaculum, a ligament attaching the testis to the bottom of the scrotum *(7,8)*. Fetal Leydig cells remain in the testis interstitium after birth, but rapidly involute *(9)*. The fate of fetal Leydig cells after puberty has been debated for many years *(10)*. The failure to resolve this issue results from a lack of markers that would allow for unambiguous identification of fetal Leydig cells as distinct from their adult counterparts. However, the fetal and adult Leydig cells can be distinguished functionally, and this may lead to an answer. Whereas luteinizing hormone (LH) stimulates development and steroidogenic function in adult Leydig cells, there is no comparable requirement for LH stimulation in fetal Leydig cells. In the LH receptor null LH receptor knockout (LHRKO) mouse, testosterone levels do not differ relative to wild-type control prenatally, and the failure to develop increased steroidogenic capacity is clearly associated with the pubertal period. These results support the hypothesis that fetal Leydig cells atrophy and become inactive in the testis postnatally *(11–14)*.

Most of the information available on the adult Leydig cell lineage comes from studies performed in the rat. The adult Leydig cells lineage first becomes evident by day 11 postpartum when spindle-shaped cells in the interstitium begin to express a functional marker, the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) *(15)*. The postnatal development of adult Leydig cells from 3β-HSD immunoreactive spindle-shaped cells is dependent in turn on a previously mysterious population of stem cells, which can be designated stem Leydig cells (SLCs). As is the case for other stem cell populations, the SLCs undergo self-renewal divisions to maintain their numbers, and have the ability to commit to lineage specific development. Stem cell commitment is followed by processes leading progressively to increased morphological and biochemical differentiation of the SLCs into adult Leydig cells that secrete testosterone and are terminally differentiated *(16)*. The regulation of these processes will be reviewed in this chapter.

Leydig Cell Ontogeny

It is undoubtedly the case that both fetal and adult generations of Leydig cells arise from stem cells, but it remains unclear in 2006 whether the mature cells in each of the generations share a common stem cell ontogeny. A shared ontogeny of Leydig cell generations is suggested by similarities of their developmental regulation. For example, the morphogen Desert Hedgehog and platelet-derived growth factors (PDGFs) induce interstitial spindle-shaped (fibroblastic) cells to express testosterone biosynthetic cholesterol side-chain cleavage enzyme on day 12.5 in the fetus, and these cells later differentiate into fetal Leydig cells *(17,18)*. Desert Hedgehog and PDGFs are also implicated in the development of the adult Leydig cell population, because targeted gene deletions suppressing Desert Hedgehog and PDGF-AA expression, prevents adult Leydig cells from forming *(19,20)*. In contrast, other lines of evidence pointing to functional differences between the two Leydig cell generations might suggest that they do not have a common origin. For example, adult Leydig cells rapidly become desensitized to bolus exposures of LH because of the presence of an inhibitory guanine nucleotide-binding protein. This protein is not expressed in fetal Leydig cells *(21,22)*,which therefore, have a more prolonged response to LH. Fetal Leydig cell steroidogenesis is independent of LH, as seen in LHRKO mice *(11–13)*. Finally, fetal Leydig cells respond to adrenocorticotropic hormone through the melanocortin type-2 receptor, whereas adult Leydig cells do not *(23)*.

The most widely held view of Leydig cell ontogeny is based on the embryological literature, and holds that mesenchymal cells of the mesonephros, originally derived from embryonic mesoderm, migrate into the testis and furnish a source of fetal Leydig stem cells (*24*; Fig. 1A). Opposed to this hypothesis is the observation that interference with the mesonephric migratory process does not perturb the eventual differentiation of fetal Leydig cells *(17)*. The fetal Leydig stem cells may alternatively move into the testis from the coelomic epithelium overlying the developing gonad *(18)*. Neural crest cells provide another potential source of stem fetal Leydig cells *(25)*. It is tempting to speculate that neural crest cells are the source of the Leydig cell lineage because a number of proteins colocalize in Leydig cells, and the brain (ref. *26;* Fig. 1B). Although fetal Leydig cells do not express the Wnt1 proto-oncogene *(18)*, a marker for the neural crest lineage, dramatic cytological transformation is typical for this lineage, and so markers may not persist throughout development. The question of ontogeny is even more poorly defined for adult Leydig cells, but a recent study proposes that these cells are derived from the neural crest lineage of neuroepithelial cells *(27)*. The evidence for this assertion is based on a regeneration model, using a cytotoxicant, ethane dimethane sulphonate (EDS) that kills the existing adult Leydig cell population which then reforms from nestin-positive cellular components lining blood vessels (Fig. 1C).

A nuclear transcription factor, steroidogenic factor (SF)-1, produced under the direction of the sex-determining region on the Y chromosome, *SRY,* directs

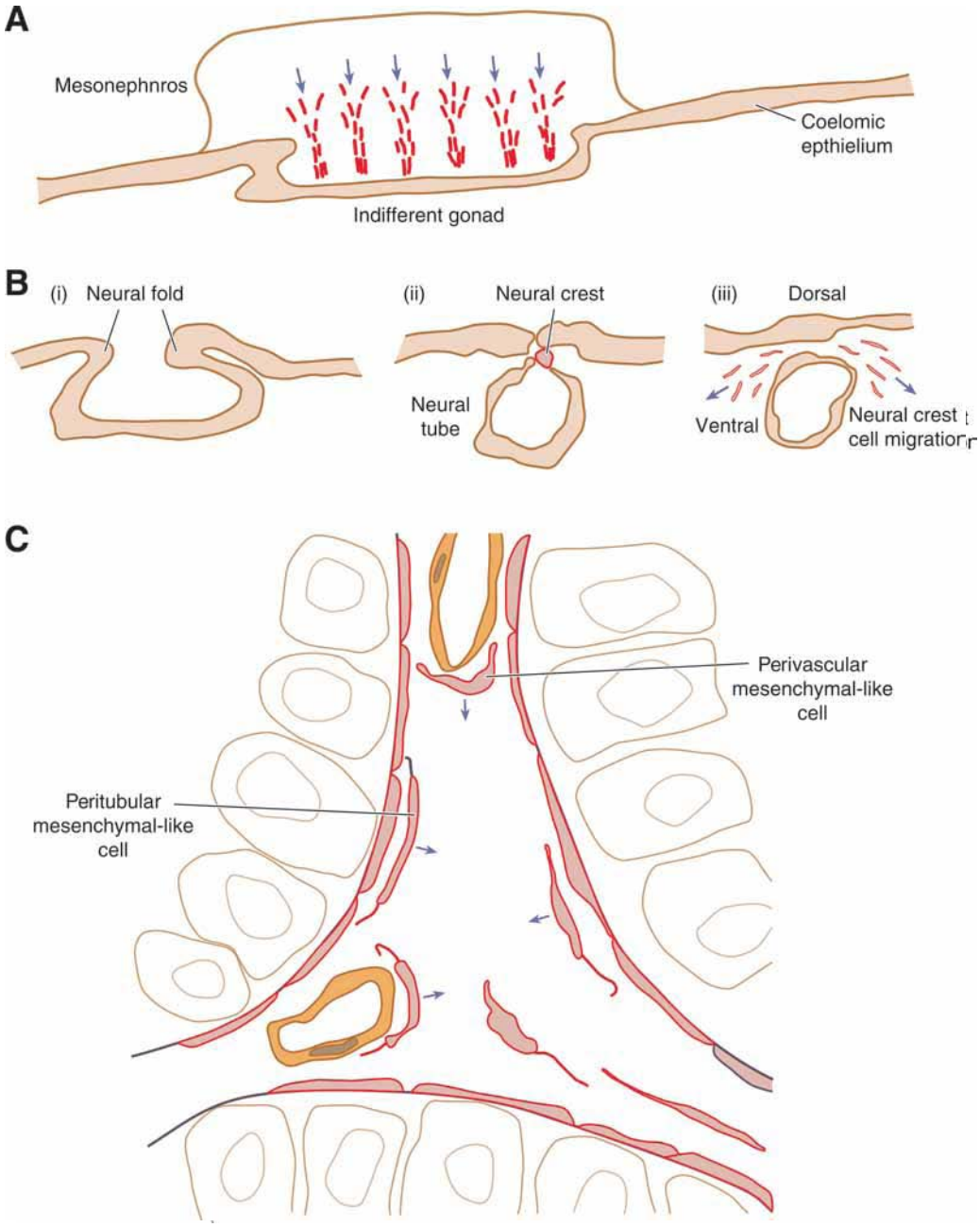


Fig. 1. Ontogeny of Leydig cells. In-migration of mesenchymal cells from the mesonephros is thought to furnish the Leydig stem cells to the interstitial spaces of the testes (arrows, **A**). However, this hypothesis has not been confirmed in studies of gonad-mesonephros host tissue recombinants, and Capel and colleagues tentatively proposed that stem Leydig cells could originate in the coelomic epithelium **(A)**. Alternatively, the pinching off of the neural tube during embryogenesis **(B)** produces neural crest cells that migrate widely throughout the body (arrows) and form several cell linages possibly including Leydig cells. However, the stem Leydig cells come to reside in the testicular interstitium, they continue to be present throughout life in peritubular and perivascular locations, with their progeny, the differentiated Leydig cells, pushed to more central locations in the interstitial space (arrows, **C**). (Please *see* color version of this figure in color insert following p. 180.)

fetal Leydig stem cells toward lineage-specific development and steroidogenic competence *(28)*. SF-1 stimulates expression of the cytochrome P450 enzymes of steroid synthesis and also promotes differentiation of Sertoli cells and pituitary gonadotropes *(29)*. The actions of SF-1 in embryonic Sertoli cells most likely include stimulating the secretion of Desert Hedgehog, PDGFs and other paracrine regulatory factors such as

insulin-like growth factor (IGF)-1 *(30)* and vasoactive intestinal peptide *(31)* that promote the differentiation and function of fetal Leydig cells. When over expression of SF-1 is induced in embryonic stem cells, or adult mesenchymal stem cells from bone marrow, these cells show the ability to differentiate into steroidogenic cells, indicating that SF-1 is involved in stem cell commitment to fetal and adult Leydig cell lineages *(28,32)*. However, further work is required to establish whether SF-1 is an essential signal for lineage commitment, because the conditional knockout of SF-1 in Leydig cells reduces, but does not entirely prevent their differentiation *(33)*.

Several types of adult stem cells have been shown to possess the ability to transdifferentiate as, for example, mesenchymal stem cells do from bone marrow. Bone marrow stem cells with mesodermal origin are able to differentiate into neurons, which are of ectodermal origin *(34)* and hepatocytes of endodermal origin *(35)*. Recently, transdifferentiation has been reported as a source of new Leydig cells in EDS-treated rat testes, with Leydig cells regenerating from vascular smooth muscle cells and pericytes of testicular blood vessels *(27)*. The concept of a mixed pool of stem Leydig cells has been suggested by Russell and colleagues, in that a multifocal origin of Leydig cells in rat testes postnatally *(36)* is discernable by electron microscopy. A schematic representation of the stem Leydig cell pool is shown in Fig. 1.

Adult Leydig Cell Ontogeny and Stem Leydig Cells

The postnatal development of adult Leydig cell population traces back to a stem cell stage. These stem Leydig cells proliferate neonatally and were identified as possible precursors largely based on the fact that they are numerous in the interstitium and, appear before adult Leydig cells are seen *(37)*. The stem Leydig cells are proliferative but are assumed not to express lineage specific markers such as steroidogenic enzymes and LH receptor *(38,39)*. They are spindle-shaped and most often located adjacent to the peritubular cells *(39,40)*. Until recently, this description would be the sum of what is known about the characteristics of stem Leydig cells. However, Lo et al. *(41)* added another potential characteristic, expression of the multidrug resistance transporter protein, in a study where Hoechst 33342 fluorescence staining was used to enrich a "Hoechst dim" side population by cell sorting. The Hoechst dim cells so obtained were seen to form testosterone producing cells after transplantation into testes of mice that have a hypoplasia of adult Leydig cells resulting from targeted deletion of the LH receptor (LHRKO) *(13)*. Because abundant expression of multidrug resistance is a characteristic of mesenchymal stem cells, this suggested that stem Leydig cells are of mesenchymal origin and Hoechst dim. Enrichment of PDGFR-α-positive cells from the interstitium of neonatal rat testes showed that these cells also express c-kit and leukemia inhibitory factor (LIF) receptor and can differentiate into androgen-producing cells in vitro *(38)*. The putative PDGFR-α^+ stem Leydig cells can be maintained in an undifferentiated state in media supplied with LIF *(38)*.

By day 11 postpartum, stem Leydig cells become committed to the Leydig cell lineage and transform into progenitor Leydig cells as judged by the onset of expression of steroidogenic enzymes and, a day later, LH receptors *(39)*. A small reservoir of stem Leydig cells persists in adult testes, supporting the concept of a slow turnover and renewal of the adult Leydig population *(42)*. Under pathological circumstances, when adult Leydig cells are destroyed by exposure to stress *(43)* or a cytotoxic chemical such as cadmium *(44)* or EDS *(45)*, the rate of Leydig cell renewal from stem cells is highly accelerated. Commitment and differentiation of stem Leydig cells into later stages of the Leydig cell lineage can be viewed as a three part process (summarized in Fig. 2): Stem to Progenitor to Immature and, finally, to adult Leydig cell.

STEM TO PROGENITOR LEYDIG CELL TRANSITION

Progenitor Leydig cells appear in the testis during days 11–28 postpartum. The progenitor Leydig cells are small, spindle-shaped cells that are identifiable as distinct from stem Leydig cells by their expression of LH receptors and steroidogenic enzymes such as 3β-HSD. At the time of commitment when progenitor Leydig cells first become distinct from stem Leydig cells they appear to express genes encoding steroidogenic enzymes such as cholesterol side chain cleavage enzyme (P450scc), 3β-HSD, and 17α-hydroxylase/20-lyase (P450c17), but *not* the LH receptor *(39)*. Despite the fact that P450scc, 3β-HSD, and P450c17 appear simultaneously in progenitor Leydig cells on day 11, evidence from LHRKO mice indicates that 3β-HSD may be the first enzyme to be induced because spindle-shaped 3β-HSD positive cells continue to exist in the interstitium of LHRKO males, whereas P450scc and P450c17 are absent *(11)*. Progenitor Leydig cells express testosterone biosynthetic enzymes at low levels, but have an abundant capacity to metabolize testosterone as 5α-reductase and

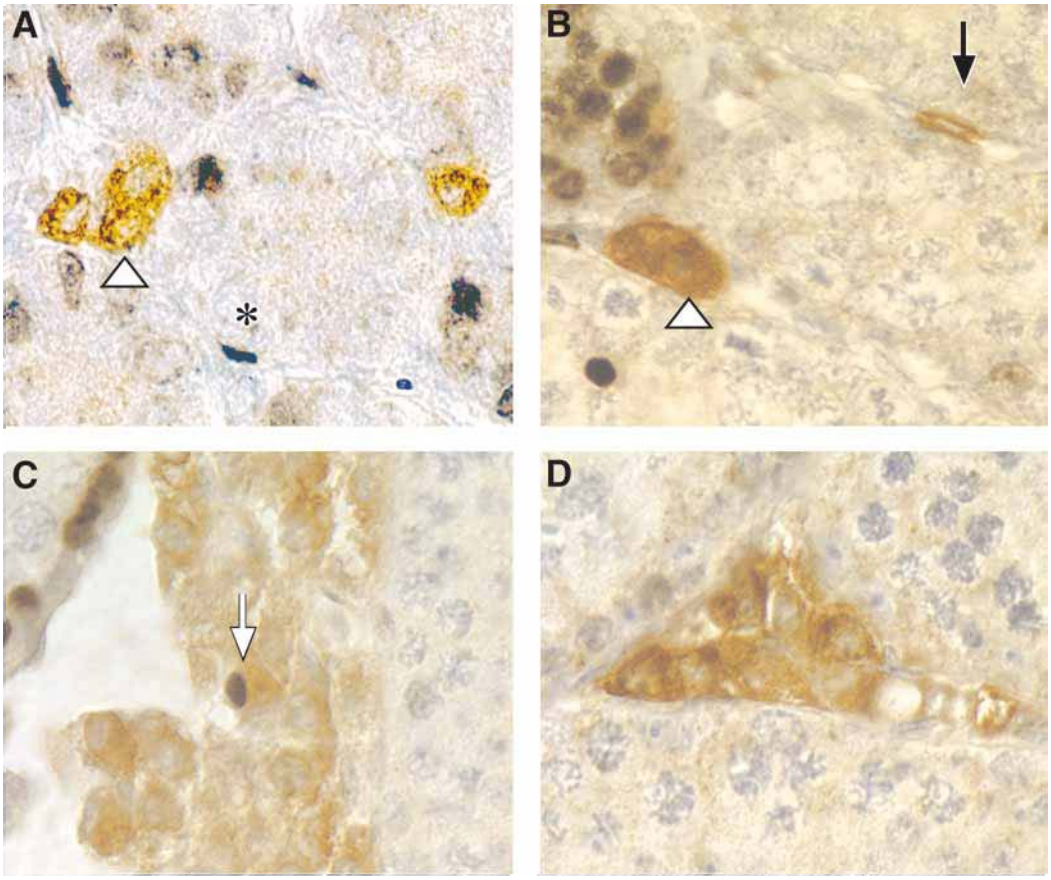


Fig. 2. Development of rat Leydig cells. Double immunolabeling of testicular cells for 3β-HSD and BrdU was assessed in sections of rat testes obtained on days 7 **(A)**, 14 **(B)**, 35 **(C)**, and 90 **(D)**. A cluster of 3β-HSD-positive presumptive fetal Leydig cells can be seen (brown staining, indicated by the white arrowheads) on days 7 **(A)** and 14 **(B)**. At this age, spindle-shaped interstitial cells, which are the putative stem Leydig cells adjacent to peritubular myoid cells (e.g., *), were often labeled with BrdU (dark blue). One week later in panel B, a spindle-shaped progenitor Leydig cell (PLC) is brown (black arrow) on day 14 **(B)**. On day 35, immature Leydig cells are apparent, and are occasionally BrDU labeled (white arrow, **C**). On day 90, adult Leydig cells have formed and are notably larger and more heavily stained by the 3β-HSD antibody **(D)**. (Please *see* color version of this figure in color insert following p. 180.)

3α-hydroxysteroid dehydrogenase levels are elevated *(46–48)*. Progenitor Leydig cells are highly proliferative and remain active in the cell cycle *(49,50)*. Their capacity for proliferation may be mediated in part by cyclin A2 expression, which is required for cell cycle-progression *(49)*. Other cell cycle progression genes are also known to be expressed at high levels in the progenitor Leydig cell, including: Cdk2, CDC25, cyclin B, cyclin C, cyclin D, and cyclin E *(50)*. Progenitor Leydig cells may retain some stem cell characteristics in that they continue to express PDGF receptor-α, LIF receptor, c-kit, which may be necessary for their proliferation until they become sensitive to LH *(38)*.

Gradually, progenitor Leydig cells enlarge, become round and their proliferative capacity is reduced. As they begin to withdraw from the cell cycle progenitor Leydig cells acquire some of the differentiated functions of mature stages of the lineage, including increased expression of P450scc, 3β-HSD, and P450c17 *(39,51)*. Paradoxically, progenitor Leydig cells contain negligible amount of smooth endoplasmic reticulum (ER), the organelle needed as a lipid platform for steroidogenic enzyme proteins, yet these cells are competent to secrete steroids. Before the smooth ER membranes become extensive, the enzymes of steroidogenesis may have other locations in the Leydig cell. For example, a mitochondrial form of 3β-HSD is known to exist in the Leydig cell although its developmental profile has not been defined *(52–54)*.

Of the enzymes needed for testosterone biosynthesis in progenitor Leydig cells, one is lacking: the 17β-hydroxysteroid dehydrogenase 3. As a consequence, the testosterone intermediate, androstenedione, is produced and rapidly metabolized to androsterone as a result of the activities of two enzymes, 5α-reductase and 3α-hydroxysteroid dehydrogenase *(48)*. Hormonal

control of the two metabolizing enzymes remains uncertain, but a large role for LH appears unlikely as progenitor Leydig cells are relatively insensitive to this hormone *(55)*. It has been established that progenitor Leydig cells express a shortened, nonfunctional form of the LH receptor *(56)* consisting only of its extracellular domain. At later developmental stage, a change in the splicing of the LH receptor gene results in the appearance of functional LH receptors *(55)*.

Only a small number of transcription factors have been found to be associated with differentiation of stem Leydig cells. It seems likely that several transcription factors will operate in concert to regulate the initial commitment of stem Leydig cells to lineage-specific differentiation. In this regard, SF-1, an orphan receptor is crucial for the development of the fetal, adrenal, and gonad. In Leydig cells, SF-1 stimulates expression of steroidogenic acute regulatory protein *(57)* needed for the transport of cholesterol from the cytosol to the inner mitochondrial membrane, and also induces P450scc *(58,59)*. A conditional knockout of SF-1 in Leydig cells leads to undetectable levels of P450scc in interstitium *(33)*. Furthermore, over expression of SF-1 in mouse embryonic stem cells or adult mesenchymal stem cells leads these cells to commit to differentiation of steroidogenic capability, forming a mixed population of adrenal and gonadal endocrine cells *(28)*. These results indicate that SF-1 is necessary but not sufficient for lineage-specific differentiation of stem Leydig cells. In this regard, it is notable that SF-1 is also highly expressed in Sertoli cells *(60)* and that Leydig cells are still present in the interstitium in Leydig cell conditional knockouts of SF-1 *(33)*. Other transcription factors that may be involved in Leydig cell development include NUR77, another nuclear orphan receptor. DNA sequence elements that bind to NUR77 are present in many 5′-untranslated regions of genes that function in Leydig cell steroidogenesis, such as 3β-HSD2 *(61)*. NUR77 achieves peak expression levels at the progenitor cell stage of Leydig cell development, providing a context for it to act together with SF-1 to regulate the commitment of stem Leydig cells *(50)*. Further upstream from SF-1, LIM-homeodomain transcription factor (Lhx9), has been implicated in various developmental processes including gonadogenesis. In the testis, Lhx9 is present only in interstitial cells *(62)*, and loss of Lhx9 in knockout mice leads to Leydig and Sertoli cell dysgenesis *(62,63)*. Progenitor Leydig cells express Lhx9, and expression levels increase as they undergo further differentiation and become competent for steroidogenesis (unpublished observations).

Progenitor Leydig cells can be purified by collagenase dispersion of the testis and density gradient centrifugation *(64)*. Following this procedure, 90% of the cells obtained from 21-d-old rats stain lightly for 3β-HSD and 75% have LH receptors *(55)*. Purified progenitor Leydig cells have been used extensively to study the regulation of Leydig cell development.

IMMATURE LEYDIG CELLS

The second transition occurs as progenitor Leydig cells continue to differentiate, producing the next intermediate, an immature Leydig cell. The immature Leydig cells are most commonly seen in the testis during days 28–56 postpartum in the rat. Freshly isolated immature Leydig cells stain intensively for 3β-HSD, have high levels of LH receptor binding *(9)* and are rounder because of increased abundance of the smooth ER *(47,55)*. In the rat, a distinguishing characteristic of immature Leydig cells is their numerous cytoplasmic lipid droplets *(47,55)*, which support a high level of steroidogenic capacity. The content of lipid droplet diminishes when these cells later mature into adult Leydig cells *(47,55)*. This transition may reflect a change in the intracellular source of cholesterol used in steroidogenesis. Esterified cholesterol from lipid droplets may be the predominant source in immature Leydig cells, whereas the source at later stages of development is, cholesterol derived from serum lipoprotein or synthesized *de novo*. The activities of three testosterone biosynthetic enzymes, P450scc, 3β-HSD, and P450c17 sharply increase during the period when immature Leydig cells are in the predominant stage: days 28 through 56 d. The rise of steroidogenic enzyme activities occurs in tandem with increases in the numbers of mitochondria and volume of smooth endoplasmic reticulum. By day 56, 17β-hydroxysteroid dehydrogenase 3 begins to be more highly expressed, catalyzing the conversion of testosterone from androstenedione to complete the androgen biosynthetic pathway *(48)*. A transient elevation of androgen metabolism occurs at the immature Leydig cell stage, and as a result of a peak in 5α-reductase and 3α-hydroxysteroid dehydrogenase (3α-HSD) activities, testosterone is converted into 5α-androstane-3β, 17β-diol (3αDIOL *[48,65]*). In addition to the 3α-DIOL-generating form of 3α-HSD, which is a reductase, immature Leydig cells express oxidative 3α-HSD activity and can convert 3α-DIOL back to dihydrotestosterone *(66)*. Retinol dehydrogenase II *(67)* has 3α-HSD oxidative activity, is present in immature Leydig cells, and might provide another source of DHT production in immature testes.

ADULT LEYDIG CELLS

Immature Leydig cells undergo a final division before adult Leydig cell function develops by postnatal day 56 *(37)*. Cell division and growth come to an end at the adult Leydig cell stage. In contrast to the earlier stages, adult Leydig cells contain a full complement of the smooth endoplasmic reticulum, few lipid droplets, high levels of steroidogenic enzyme activity, and secrete testosterone as the predominant androgen end product. A dramatic shift between production of 3α-DIOL and testosterone results as the immature Leydig cells differentiate into adult Leydig cells and 5α-reductase expression falls off sharply *(48)*. Adult Leydig cells comprise the majority of the Leydig cell population of sexually mature testes, but smaller numbers of the precursor stages continue to be present in adulthood. Cell replication among the precursor stages, may continue to occur during adulthood, but is slow after puberty *(68)*. In fact, the estimated turnover time for adult Leydig cells exceeds the 2-yr lifespan of the average rat *(68)*. A balance between cell replication by the precursor stages and apoptosis by the adult Leydig cell apoptosis maintains a constant Leydig cell number per testis—approx 25 million *(69)*. Environmental toxins, stress, and seasonal breeding cycles increase the rate of apoptosis in Leydig cells and, also their turnover *(70–73)*.

The population of adult Leydig cells is achieved during postnatal development, starting with stem Leydig cells present in the interstitium at birth. Stem Leydig cells divide asymmetrically, forming one daughter stem Leydig cell and a daughter committed cell, which will give rise to a progenitor Leydig cell. Leydig cells are generated thereafter through a combination of progenitor Leydig cell proliferation, departure of the progenitor and immature Leydig cells from the cell cycle, and differentiation and functional maturation of the progenitor and immature Leydig cells into adult Leydig cells. Stem, progenitor, immature, and adult Leydig cells have distinct sets of biochemical and morphological characteristics and respond differently to hormonal regulators.

HORMONAL REGULATION OF LEYDIG CELL DEVELOPMENT

Leydig cells are exposed to multiple regulatory factors that precisely control their numbers and steroidogenic capacity. The gonadotropin, LH, has a pre-eminent critical role in Leydig cell differentiation, once LH receptors are expressed. However, at the earliest stem cell stage when LH receptors are not present, other regulatory factors must be active, and many of these are thought to originate in the local environment of the testis, from Sertoli and peritubular myoid cells, testicular macrophages, and possibly the Leydig cells themselves.

Leukemia Inhibitory Factor and Interleukin-6

LIF, a member of the interleukin (IL)-6 family of cytokines, exerts its effects by binding to a heterodimeric receptor made up of a LIF-specific binding subunit (gp190) coupled to a transmembrane signal transducing subunit (gp130) receptor chain, which also is used as the receptor subunit for IL-6 *(74)*, oncostatin M *(75)*, cardiotrophin-1 *(76)*, and ciliary neurotrophic factor *(77)*. LIF is essential for blastocyst implantation and the normal development of hippocampal and olfactory receptor neurons. LIF has been used extensively to induce embryonic stem cells to retain their totipotency. LIF signaling leads to activation of the JAK/STAT (Janus kinase/signal transducer and activator of transcription) and mitogen-activated protein kinase cascades *(78)*. LIF is required for long-term self-renewal of neural stem cell cultures *(79)* and for maintenance of primordial germ cell cultures *(80)*. In the rat testis, LIF is detectable from 13.5 d of gestation onward, and is predominantly expressed by the peritubular cells surrounding the seminiferous tubules *(81)*. In the 1st wk postnatally, the peritubular cells have a fibroblastic ultrastructure *(14)* and form a two to three cell layer-thick boundary tissue (aka, lamina propria). It is likely that the stem Leydig cells are situated in the outermost layer of the boundary tissues, in the interstitial space *(14)* and therefore, are likely targets of LIF signaling. Stem Leydig cells and all cells of the Leydig cell lineage express gp130, with the highest levels of the protein being in stem and progenitor Leydig cells *(38,50,82)*. The action of LIF on differentiated Leydig cells results in decreased steroidogenesis *(83)*, which occurs in part by reducing the availability of cholesterol substrate to P450scc in the mitochondria *(82)*. Given that LIF withdrawal is a stimulus for differentiation of mouse embryonic stem cells *(84)* a similar action may apply in the case of stem Leydig cells. Consistent with this idea, LIF has been found to stimulate stem Leydig cell proliferation *(38)*. IL-6, which is closely related to LIF, is more highly expressed in immature testis relative to the adult, and is present in Sertoli cells, testicular macrophages, and Leydig cells *(85–88)*. The IL-6 receptor is expressed in progenitor, immature and adult Leydig cells *(85,89)*. Therefore, both LIF and IL-6 may regulate stem Leydig cells and other early members of the Leydig cell lineage. The LIF knockout mouse does not have a pronounced male reproduction phenotype, but this may be ascribed to substitution by other related proteins (IL-6). The LIF

receptor knockout is lethal to animals in the perinatal period *(90)*, and it will be necessary to create conditional knockouts of the receptor in Leydig cells to study its developmental effects further.

Platelet-Derived Growth Factors

PDGFs form a family of disulfide-linked homodimeric proteins that exert mitogenic effects on undifferentiated mesenchymal cells in early stage embryos and progenitor cell populations. They are also implicated in tissue remodeling and differentiation. PDGF receptor-α signaling is essential for differentiation of fetal Leydig cells *(18)* and stimulates differentiation of adult Leydig cells as well. PDGF receptor-α is expressed both in the Leydig cell lineage and peritubular myoid cells *(20,50,91,92)*. It has been established that adult Leydig cells do not differentiate in knockout mice which lack expression of the PDGF-AA ligand (the homodimer); *(93,94)*. Expression of PDGF in peritubular cells begins before birth, well ahead of the onset of Leydig cell-specific genes such as LH receptor *(95)*, and thus, PDGF receptor-α signaling may be a proximate cause of commitment of stem Leydig cells to Leydig cell differentiation.

Kit Ligand Kit receptor is present on type A1 spermatogonia *(96)* and in Leydig cells *(97–100)*. Interstitial expression of Kit is first detectable by day 7 postpartum in the mouse *(97)*. Kit is expressed at higher levels in progenitor Leydig cells relative to immature and adult Leydig cells *(50)*. Kit ligand (also known as stem cell factor) is produced by the putative stem Leydig cells *(38)* in addition to Sertoli cells *(98,101)*. Given that Kit ligand stimulates germ cell proliferation and survival, an analogous role for this factor has been postulated for Leydig cells. The signaling functions of Kit are mediated by receptor autophosphorylation and subsequent association with PI 3-kinase. To investigate the role of Kit-mediated PI 3-kinase signaling in vivo, a knockin mouse, Kit^{Y} 719^{F}/$Kit^{Y}719^{F}$, was created containing a tyrosine-to-phenylalanine substitution mutation located in the canonical binding site for the p85 subunit of PI 3-kinase *(102)*. This mutation causes Leydig cell hyperplasia in adult testes *(102)*, indicating that Kit regulates Leydig cell proliferation and, possibly, differentiation. The effects of the $Kit^{Y}719^{F}$ mutation on Leydig cell development and steroidogenic function were investigated and reduced testosterone biosynthetic capacity was observed *(103)*. Kit ligand stimulates testosterone production through the PI3-kinase pathway, as seen by the fact that the stimulatory action was not detected in Leydig cells of the $Kit^{Y}719^{F}$ knock-in mice *(103)*. Further support for a developmental role of Kit ligand was obtained in a study using an antibody directed against the Kit receptor, which partially blocked Leydig cell regeneration in EDS-treated rats *(104)*.

GONADOTROPINS (LH AND FSH)

LH is the major stimulus regulating testosterone synthesis in Leydig cells and, although there is evidence for LH receptor expression in testicular capillaries *(105)* and the epididymis *(106)*, LH action has been most clearly delineated in this cell type *(107)*. LH binding to its receptor triggers the cAMP signaling cascade leading to rapid effects, including cholesterol mobilization *(108–110)*, and elevated steroidogenic enzyme activity, and longer term transcriptional effects. Cessation of LH signaling eventually, results in loss of steroidogenic enzyme activities, and declines in steroidogenic organelle volume and numbers and general cell atrophy *(111)*.

LH stimulation is required for Leydig cell development, but it is unlikely to be the initial stimulus for stem cell differentiation into the Leydig cell lineage or the trigger for initial expression of Leydig cell-specific genes. Evidence for this assertion comes from the fact that the LH receptor protein is truncated in progenitor Leydig cells, providing an attenuated response to gonadotropic stimulation *(56)*. That LH plays a critical role in the development of Leydig cells is apparent from studies of $GnRH^{hpg}$ mice, which are deficient in circulating LH. In these mice, Leydig cell numbers are about 10% of control *(112)*. Leydig cells are also severely hypoplastic in LHRKO mice *(13,113)*. In normal rats and mice, increased Leydig cell proliferative activity occurs following LH/human choriogonadotropin (hCG) administration in vivo *(69,108,114)*, although the underlying mechanism subsequent to LH receptor binding that leads to cell cycle progression has not been identified. In adult Snell dwarf mice, a deficiency in plasma gonadotropin prevents full differentiation of Leydig cells without affecting their numbers *(115)*. Neither long-term suppression of LH nor the return of LH to control values in adult rats has a significant effect on Leydig cell numbers *(116,117)*. In addition, although LH stimulates DNA synthesis in immature rat Leydig cells in vitro, these increases are limited; significant enhancement of the LH effect is achieved by coadministration of growth factors such as IGF-1 *(49,118)*. These results raise the possibility that the action of LH on Leydig cell proliferation requires the participation of, or is preceded by, the action of other factors.

Follicular stimulating hormone (FSH) stimulates functions in Sertoli cells, but this may in turn act on Leydig cells indirectly. The evidence for FSH action on Leydig cell development is equivocal. Administration of FSH injections to animals with low or absent circulating LH stimulates Leydig cell differentiation, and steroidogenic activity *(119–124)*. Similarly, an inactivating mutation of the FSH receptor reduces the numbers of Leydig cells, and their steroidogenic capacity *(125)*. However, in the presence of normal LH levels, FSH action is not required and, consistent with this idea, the FSH-β null mutation has no discernible effect on Leydig cell numbers *(125)*. This contrasts with the GnRH-deficient mouse lacking both LH, and FSH which fails to develop Leydig cells *(112)*.

OTHER GROWTH FACTORS

Of the growth factors that have been analyzed in conjunction with Leydig cell development, IGF-1, has been the most studied *(126)*. IGF-1 mRNA, protein, and receptors have been identified in Leydig cells, peritubular cells, and spermatocytes *(127–132)*. Testicular levels of IGF-1 are the highest at 4 wk postpartum, at the beginning of the pubertal rise in testosterone secretion *(133)*. LH and hCG stimulate IGF-1 secretion and upregulate type-1 IGF-1 receptor gene expression in rodent Leydig cells *(133–136)*. IGF-1 stimulates the proliferation of Leydig cell precursors, and pretreatment of these cells with LH augments the mitogenic effect *(49,118,132)*. IGF-1 facilitates Leydig cell differentiation and maturation in conjunction with LH. Other factors such as IGF-1 must stimulate development and fulfill a stimulatory role not provided by LH based on the observation that progenitor Leydig cells possess few LH receptors and are relatively insensitive to LH *(55,137)*. Second, Leydig cells differentiate in $GnRH^{hpg}$ mice *(112)* despite the deficiency of circulating LH in this line. Third, IGF-1 and its receptor mRNAs are highly expressed in progenitor and immature Leydig cells, and IGF-1 is known to enhance hCG-stimulated testosterone formation *(138)*. This suggests that there is a requirement for IGF-1 that precedes LH-mediated differentiation of the Leydig cell and that IGF-1 acts in conjunction with LH to further stimulate the maturational process.

In vitro studies have shown that IGF-1 stimulates maturational events such as increased expression of steroidogenic enzymes leading to higher rates of testosterone production *(139,140)*. In contrast, the GH-deficient Snell dwarf mice have negligible circulating IGF-1, and low testosterone levels. Administration of IGF-1 to these mice in vivo induces a marked increase in the numbers of LH receptors and in the steroidogenic response *(141)*. It has been shown previously that mice with an IGF-1-null mutation have marked reductions in circulating testosterone levels (18% of wild-type control), associated with decreases in testis size and Leydig cell numbers *(142)*. This led to the hypothesis that the dramatic declines seen in circulating testosterone levels in adult IGF-1-null mutants result from abnormal testis development, and specifically from an imbalance in testosterone biosynthetic and metabolizing enzyme activities in Leydig cells.

It has become clear that although IGF-1 is important for the development of normal steroidogenic competence, it is not active at earlier points in the differentiation pathway. For example, a lack of IGF-1 signaling in the knockout mouse does not completely prevent Leydig cells from forming. It is possible that IGF-1 is not entirely eliminated in the male pups during the perinatal period, because IGF-2 is present at that time and is known to bind the IGF-1 receptor *(143)* but the results suggest that other factors act proximal to IGF-1 signaling.

ANDROGEN

Androgen receptor is present at all stages of the Leydig cell lineage *(55,144–147)*, but the trend is for higher expression levels in progenitor, and immature compared with adult Leydig cells *(55,148)*. The presence of androgen receptors indicates that androgen directly regulates Leydig cell development and function. When cultured for 3 d in the presence of LH and dihydrotestosterone, progenitor Leydig cells increase their capacity for testosterone production more than 10 times and undergo cytological differentiation *(64)*. The presence of numerous androgen receptors in progenitor Leydig cells, which have few LH receptors, indicates that androgen action may precede and facilitate the response to LH *(55)*. Exposure of progenitor Leydig cells to androgen stimulates increases in the protein levels of LH receptor, androgen receptor, and 3α-hydroxysteroid dehydrogenase *(148)*. In mice with naturally occurring mutations causing androgen insensitivity, designated testicular feminization, Leydig cell numbers are decreased and differentiation of Leydig cells is incomplete *(149–151)*. The defect could arise indirectly, because the testes fail to descend and the elevated temperature associated with cryptorchidism is known to affect Leydig cells, or as a secondary consequence of deficient androgen action on Sertoli cells. Sertoli cells also contain androgen receptors *(152–154)* and the Sertoli cell-specific knockout of androgen receptor

reduces Leydig cell numbers by 40%, as opposed to 83% in mice with a knockout of the androgen receptor in all tissues *(155)*. The fact that the reductions are less severe in the Sertoli cell conditional compared with the total knockout points to a role for androgen receptor signaling in the Leydig cell *(155)*.

ESTROGEN

Two types of estrogen receptors (ERs)-α and -β have been identified, and the α-form is the primary subtype in Leydig cells. ER-β is detected in mouse Leydig cells *(156–158)*, in which its function remains poorly understood. In ER-αKO mice, Leydig cell steroidogenic capacity is increased, but this may simply be a consequence of elevated LH levels resulting from a decline in estrogen-negative feedback on the pituitary gonadotropes *(159)*.

DESERT HEDGEHOG

The Hedgehog signaling pathway is involved in a number of developmental processes during embryogenesis. At least three Hedgehog proteins, Desert, Sonic, and Indian, so far have been identified and they act through the patched receptor. Two patched receptors, patched 1 and 2, when not bound by Hedgehog proteins, repress the action of Smoothened *(160)*, Smoothened is a transmembrane protein mediating the Hedgehog signal that induces upregulation of the transcription factor Gli *(161)*. Desert hedgehog (Dhh) is expressed by preSertoli cells in the embryonic testis *(162)*, and its regulation is closely tied to the action of testis-determining factor *Sry*. Dhh knockout male mice are sterile, and development of adult Leydig cells is defective *(19)*. The patched receptor is present in Leydig cells *(19)*, which is consistent with a requirement for Dhh activity in the development of androgen synthesis. Dhh also appears to act in the fetal testis, and the knockout prevents fetal Leydig cells from forming. Migration of putative fetal Leydig stem cells from the mesonephros and their proliferation and survival in the interstitium were unaffected in the Dhh knockout, and the defect is presumed to lie with some aspect of differentiation *(17)*.

ROLE OF TESTICULAR MACROPHAGES

Macrophages and Leydig cells exist in close proximity in the testicular interstitium *(163)*. Macrophages also secrete cytokines such as IL-1 and transforming growth factor-α that stimulate proliferation of progenitor Leydig cell *(164,165)*. Developmental interactions between macrophages and Leydig cells have been noted in osteopetrotic mice, which have few macrophages as a result of a null mutation in the gene encoding colony-stimulating factor-1 (*op/op*). Leydig cells obtained from *op/op* males are deficient in steroidogenic enzymes expression and testosterone production *(166)*. In neonatal and immature rats, when macrophages are depleted from the testis following treatment with dichloromethylene diphosphonate, adult Leydig cells fail to develop normally *(167–170)*. These results indicate that the dendritic cell and Leydig cell lineages are developmentally coupled, although the evolutionary advantage conferred by their association remains to be defined.

CONCLUSIONS

Postnatal development of adult Leydig cell population draws upon a pool of stem Leydig cells, which proliferate and commit to the Leydig cell lineage. It is unknown at the present time whether the stem Leydig cells are unipotential or pluripotential, and their ultimate ontogeny in the embryo is a topic of continued investigation. Amplification of Leydig cell numbers occurs primarily during the progenitor stage, and a small number of Leydig stem cells continue to exist in the testis throughout adult life. The morphological and biochemical characteristics of the intermediate stages of Leydig cells have been defined, providing increased understanding of the factors that control the development of steroidogenic capacity. After commitment of stem Leydig cells to the Leydig cell lineage, there are two distinct stages of intermediate development. The first is a spindle shaped progenitor Leydig cell that contains steroidogenic enzymes such as P450scc, 3β-HSD, and P450c17, and has truncated LH receptors on the cell surface. The progenitor Leydig cells also express metabolic enzymes such as 5α-reductase, and 3α-hydroxysteroid dehydrogenase, and androsterone is their primary androgen end product. The progenitor Leydig cells differentiate into a second intermediate, the immature Leydig cell, during days 14–28, and these cells primarily produce 3α-DIOL. On day 28, there are on average 12 million immature Leydig cells per testis, which undergo one further round of mitosis and differentiate into adult Leydig cells by day 56.

The transition from proliferating progenitor Leydig cell to differentiated adult Leydig cell is hormonally regulated. The transcription factors responsible for commitment of stem into progenitor Leydig cells are largely unknown. SF-1, NUR77, and Lhx9 may be involved, but additional factors will assuredly be identified in the next few years. Growth factors produced

pre-eminently, although not exclusively, by Sertoli cells include Desert Hedgehog, PDGF, LIF, Kit ligand, and IGF-1 and may act sequentially or together to regulate the early transitions from stem to later stage Leydig cell before LH sensitivity is acquired. Androgen, potentially secreted by fetal Leydig cells may be essential for initial development of adult Leydig cells. LH signaling is necessary to amplify cell numbers further and induce the differentiation of later stage Leydig cell intermediates. The net result of the developmental process of puberty is the creation, in the testis of the adult rat, of a population of about 25 million Leydig cells that produce testosterone required for spermatogenesis and pubertal masculinization.

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GLOSSARY

Adult Leydig Cell

A terminally differentiated Leydig cell. Adult Leydig cells have an extensive smooth endoplasmic reticulum, a distinct rim of heterochromatin in the nucleus, prominent nucleolus and few lipid droplets. They primarily produce testosterone.

Fetal Leydig Cell

A terminally differentiated Leydig cell in the fetus. Fetal Leydig cells originate in the testis during gestation. They have an extensive smooth endoplasmic reticulum and, in the rat, many lipid droplets. The fetal Leydig cells produce testosterone and IGF-3.

Leydig Cell

A testosterone-producing cell in the interstitium of the testis.

Progenitor Leydig Cell

A cell that is produced by the commitment of a stem Leydig cell to the Leydig cell lineage. Progenitor Leydig cells remain fibroblastic in appearance but posses Leydig cell specific markers such as 3β-HSD and LH receptors.

Stem Leydig Cell

The founder cell of the Leydig cell lineage. A stem Leydig cell is unique in the Leydig cell lineage, in that, it divides to produce two daughter cells with different fates. One of the daughter cells is a stem cell identical to the mother cell. The other daughter is a progenitor Leydig cell that will divide, amplifying its numbers, eventually, differentiating into an adult Leydig cell. However, this asymmetric division of stem Leydig cells has yet to be observed.

Immature Leydig Cell

An intermediate in the Leydig cell lineage during postnatal development, derived from a progenitor Leydig cell. Immature Leydig cells are similar to fetal Leydig cells morphologically, in that both possess numerous lipid droplets. However, fetal Leydig cells have low 5α-reductase activity and secret testosterone, whereas immature Leydig cells abundantly express 5α-reductase, and 3α-hydroxysteroid dehydrogenase and primarily produce androstane-3α, 17β-diol.

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5 The Human Leydig Cell

Functional Morphology and Developmental History

Frederick P. Prince, PhD

SUMMARY

This chapter provides an overview of the functional morphology of the mature human Leydig cell with emphasis on the adult developmental phase. The morphology of the organelles involved with steroidogenesis is described and correlated with pertinent literature on the location of the enzymes involved. A second major topic revolves around cholesterol uptake, transfer and storage within the cell, and delivery to the mitochondria. Morphological correlates include the endosomal system, lysosomes, microperoxisomes, lipid droplets, mitochondrial associated membranes, and lipid-associated membranes. The described intimate associations of these various intracellular structures suggest extensive membrane fusion being the mechanism of cholesterol transfer and delivery to the cristae compartment of the mitochondria. A revised structural analysis of mitochondria in these steroid-producing cells is included, as well as previously unpublished evidence of a continuity of microperoxisomes with the smooth endoplasmic reticulum. The emerging picture from these transmission electron microscopy images is an organelle assemblage which is highly dynamic, with membrane fusion likely being an important aspect of cellular function in steroidogenesis. Other ultrastructural features of Leydig cells are discussed, including "neuronal features." A brief overview of the triphasic nature (fetal phase, neonatal phase, and adult phase) of Leydig cell development in human is provided, as is a description of the immature Leydig cells of childhood.

Key Words: Development; human; Leydig cell; organelles; steroidogenesis; ultrastructure.

INTRODUCTION

Interest in the Leydig cell has increased exponentially since the first description by Leydig in 1850 *(1)*. The early histological and biochemical literature has been well covered in other sources *(2,3)*. Excellent reports on Leydig cell ultrastructure in human and numerous other species are available *(2–7)*, as well as reviews on various aspects of the cell biology of these steroid-producing cells *(8–13)*. Christensen's 1975 review of human Leydig cell structure is a cornerstone in the study of the functional morphology of this cell.

This chapter will summarize the functional morphology of the human Leydig cell. Leydig cell morphology reflects the state of the hypothalamic-pituitary-testicular axis, the developmental phase of the cell, local paracrine influence, and changes in response to various other hormonal and physiological influence. The major intention of the chapter is to describe the ultrastructural features of the fully developed human Leydig cell. The morphology of the adult Leydig cell (ALC) will be the focal point; however subtle differences seen between the mature adult and neonatal Leydig cells are included. Emphasis will be placed on the organelles involved in steroid synthesis and the delivery of cholesterol to this synthetic apparatus. The hope is to provide a morphological framework in which to interpret the biochemistry of steroidogenesis. Other notable features of the Leydig cell will also be discussed. Finally, an overview of the developmental history of Leydig cells in humans will be provided.

FUNCTIONAL MORPHOLOGY OF THE HUMAN LEYDIG

General Overview of the Ultrastructure of the Mature Human Leydig Cell

Smooth endoplasmic reticulum (SER), the hallmark organelle in steroid-producing cells, is ubiquitous, as seen in the low power micrographs (Figs. 2 and 3).

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Mitochondria are numerous and rather homogeneously distributed throughout the cytoplasm. Microperoxisomes (MP) are also well distributed, but because of their small size and moderate electron density, not obvious at low magnification. Lysosomes of varying degree of maturation are numerous and quite eclectic in morphology, but are generally not as uniformly spaced as the mitochondria. Lipid droplets also vary in frequency in cellular sections and tend to be present in focal regions. The Golgi elements are well developed, often found in a "central" area surrounding the centrosome. Mitochondria and secondary lysosomes, as well as multivesicular bodies (MVB) (early and late endosomes) and assorted smooth and coated vesicles (CV) are also in close proximity to the Golgi region. These general ultrastructural features are demonstrated in the low magnification micrographs and summarized in the diagram (Fig. 1). Specifics on the ultrastructure of these organelles follow in the subsequent sections. Recent information on the internal structure of mitochondria and evidence of a continuity of the MP with the SER is included.

Organelles of Steroidogenesis

The dominant organelle of the Leydig cell is the SER. In well-preserved cells, either through fixation by perfusion or immersion, the SER is found in the form of anastamosing tubules in human Leydig cells *(3–5)*. This is quite impressive in transmission electron microscopy (TEM) sections, which exhibit extensive profiles of the tubules in cross-section and "longitudinal" section (Figs. 2 and 3). Cisternal forms (with fenestrations), which are common in a number of species *(2,6)*, are essentially not present in the adult human Leydig cell. Also, extensive areas of SER in the form of "whorls," a feature of some rodents *(3)*, are not observed in the developed human Leydig cell. In contrast, layered fenestrated cisternae of SER are often present in the immature Leydig cells of childhood (subsequent section; Figs. 14 and 15), as well as in regressing Leydig cells after the neonatal developmental peak. Thus, there exists a striking correlation of the anastomosing tubular form of SER with highly active steroid production in human Leydig cells.

The SER contains membrane bound enzymes, which are integral in steroidogenesis. Early biochemical studies done on the microsomal fraction from cell fractionation studies demonstrated the function of these membranes in steroid synthesis *(14,15)*. More recent studies using techniques of immunolabels with electron microscopy have more precisely identified the SER as the prime site of activity of these enzymes *(16–20)*. Testosterone production has also been directly correlated with SER volume percent through electron microscopic morphometric studies on Leydig cells and adrenal cortical cells *(21–24)*.

The steroid synthetic pathway begins with the conversion of cholesterol to pregnenolone on the cristae membranes of the mitochondria. P450 cholesterol side chain cleavage enzyme, which catalyzes this reaction, and it has been localized to the cristae membranes in various steroidogenic cells *(25–29)*. The delivery of cholesterol to the cristae will be discussed in a subsequent section.

Generally, mitochondrial profiles seen in thin sections are spherical, oval, or elongate and approx 0.5 µm in width. This is perhaps misleading because much evidence indicates a linear and/or reticular structure in various cell types *(30–32)*. A similar structure is also likely the case in Leydig cells as branching profiles are present. The internal structure of mitochondria of steroid-producing cells has long been considered different from those of "typical," nonsteroid-producing cells *(33)*. Although cristae structural diversity has been described in both "types" of mitochondria (reviewed in refs. *34,35*) the general description of mitochondrial cristae from typical cells as lamellar and those from steroid cells as tubular has been dogmatic until very recently.

During the past few years there have been significant advances made regarding to the basic structure of mitochondria. The technique of electron microscopic tomography has championed this revision with studies on mitochondria from a variety of cell types from nonsteroid producing cells *(36–41)*. The new paradigm of mitochondrial structure revolves around the fact that cristae originate from the inner mitochondrial membrane (IMM), perhaps more appropriately termed the inner boundary membrane (IBM), through roughly circular connections of approx 30 nm outside diameter, termed crista junctions by Perkins *(38)*. Thus, rather than having extensive folds which connect to the IBM, the cristae membranes made up of a more isolated, distinctive intraorganellar compartment. This morphology, of course, is more in harmony with the function of oxidative phosphorylation through the chemiosmotic mechanism, which depends on a significant proton gradient across the cristae membrane. A rather intriguing aspect of this revision in mitochondrial structure is that two excellent TEM studies done in the early years of electron microscopy had described them correctly *(42,43)*, but the "picture" of folded cristae became dogma. Recent studies *(44,45)* further implicate a functional difference between the cristae and the IBM with oxidative phosphorylation being more localized to the cristae. Thus, it is most accurate to describe mitochondrial structural components as the outer mitochondrial membrane (OMM), IBM, cristae membranes, and the matrix.

Regarding the mitochondria of steroid-producing cells, recent studies from this lab *(46–48)* have shown the pleomorphic nature of the cristae, demonstrated

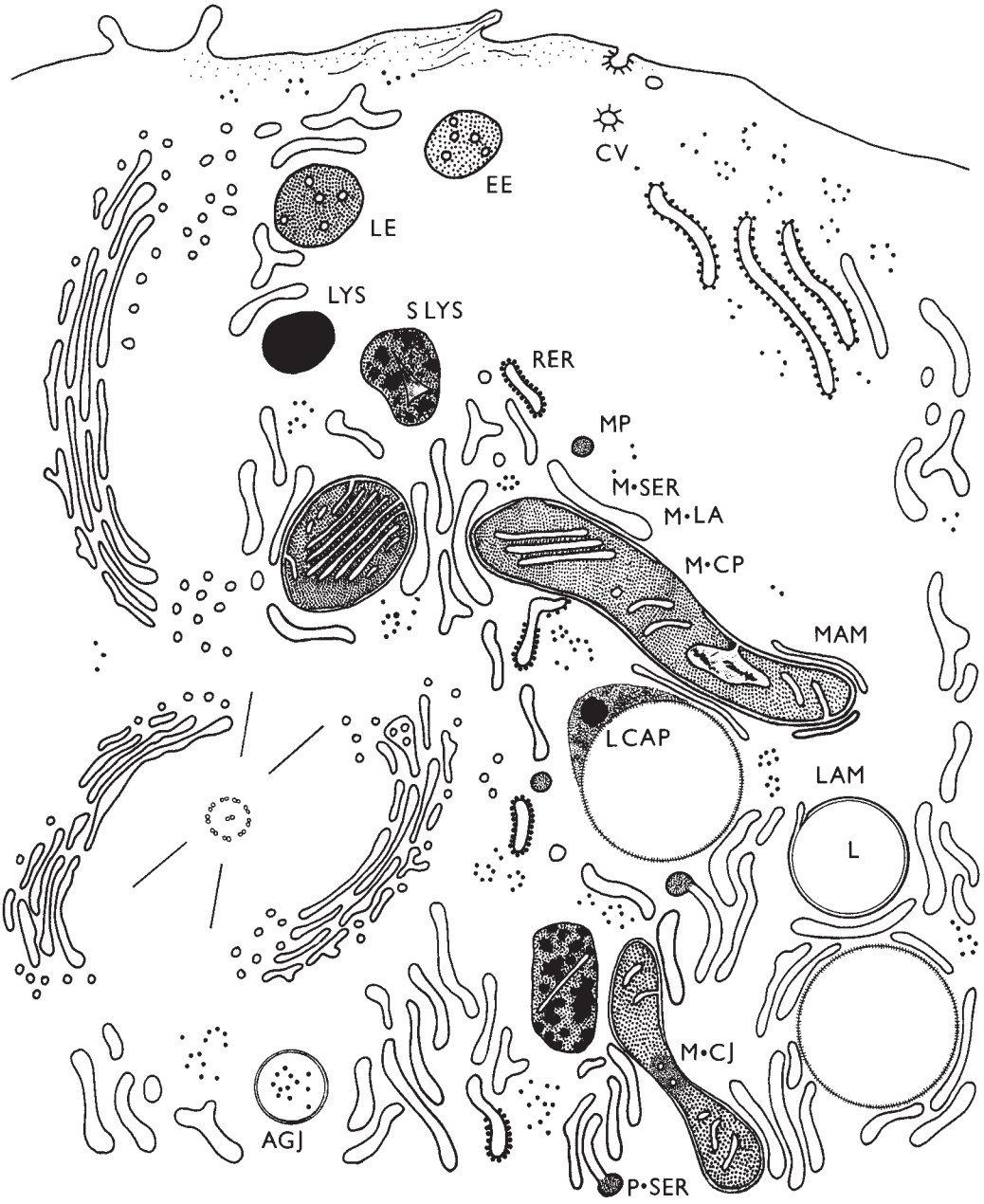


Fig. 1. This diagram provides an overview of the organelles and other structural features which are related to steroid synthesis and delivery of cholesterol to the mitochondria. The smooth endoplasmic reticulum (SER) and Golgi elements are not labeled. The top half of the diagram summarizes the uptake of cholesterol via LDL and transfer of cholesterol to the mitochondria. Low density lipoproteins are taken into the cell through receptor mediated endocytosis. Coated pits and coated vesicles are the morphological correlates. Early endosomes, then late endosomes are involved in the subsequent transfer of cholesterol to lysosomes, which mature into secondary lysosomes. The Golgi elements are involved with production of lysosomes, as well as cholesterol and vesicular transport. Lysosomes mediate, by mechanisms yet unclear, the transfer of cholesterol to the mitochondria. The mitochondria and SER contain the enzymes of the steroidogenic pathway from cholesterol to testosterone. Morphological features of mitochondria include the outer membrane, inner boundary membrane (IBM), matrix, and cristae. The cristae open to the IBM through narrow circular junctions termed crista junctons (M CJ), as shown in the lower right. Also of functional significance, there exists a close association of the mitochondria with the SER (M SER) and thin mitochondrial-associated membranes (MAM). Contact points (M CP), regions of fusion of the outer mitochondrial membrane and IBM are important in the translocation of cholesterol to the cristae membranes. A substructure of the cristae is the lamellar association (M LA), unique to steroid-producing cells and function unknown. The lower right area of the diagram depicts the variablity of lipid droplet morphology and another source of cholesterol for steroidogenesis. Lipid droplets contain stored cholesterol ester which can be converted to free cholesterol and transferred to the mitochondria. Structures likely involved are the MP, lysosomes, LAMs, and "lipid caps" (L CAP). A third source of cholesterol is *de novo* synthesis involving SER and MP. The continuity of MP with the SER is also indicated (P SER). AGJ, internalized annular gap junction.

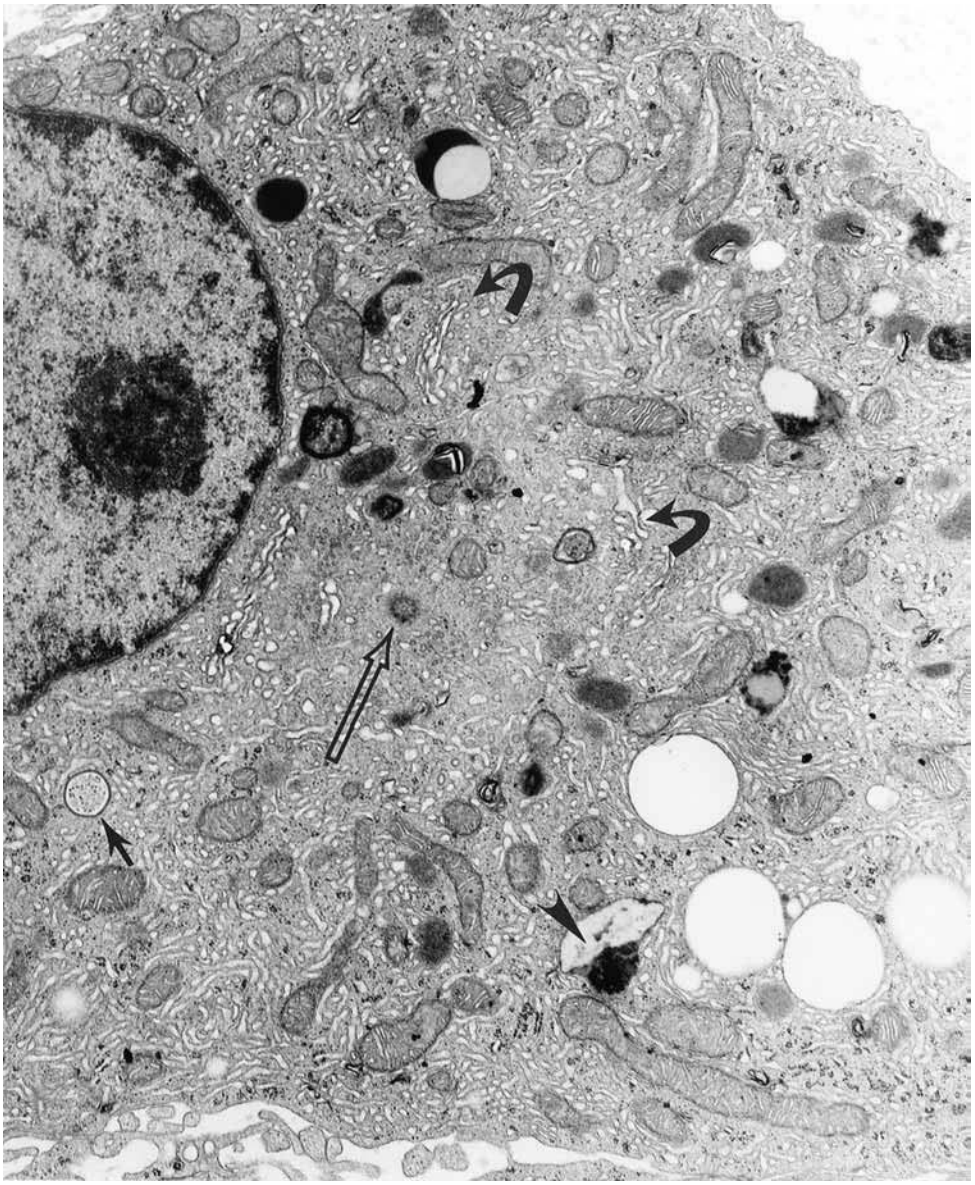


Fig. 2. Low power electron micrograph of a human Leydig cell of the adult maturation phase exhibiting many of the features summarized in the diagram. Multiple Golgi elements (two are indicated by curved arrows) surround the centrosome region (one centriole is indicated by the open arrow). The extensive network of smooth endoplasmic reticulum is evident. Mitochondria and lysosomes are common. One secondary lysosome is shown by the arrowhead. Lipid droplets are variable in number in human Leydig cells, often in localized groups, as the four in this section. Black arrow—internalized annular gap junction ×15,000.

that the cristae connect to the IBM by crista junctions as in other mitochondria and noted contact points of varying size. The roughly circular crista junctions are evident in tangential sections of the IBM in TEM sections as described previously *(46)* and shown in Fig. 4. Three dimensional models of human Leydig cell mitochondria through electron microscopic tomography provide graphic evidence of this aspect of mitochondrial structure *(48)* (*see* color plate). Also, recently come to the attention of the author, an early freeze-fracture study of the guinea pig adrenal cortex demonstrated circular profiles where the cristae connected to the IBM *(49)*. Although the vast majority of the cristae from the adrenal cells are tubular (often referred to as tubulovesicular), a few lamellar cristae were noted to also connect through circular openings to the IBM.

An intriguing subcomponent of the cristae, described as the lamellar association (LA), consisting of closely apposed lamellar cristae, is present in mitochondria from human Leydig cells *(46–48)* (Fig. 4 and color plate). This

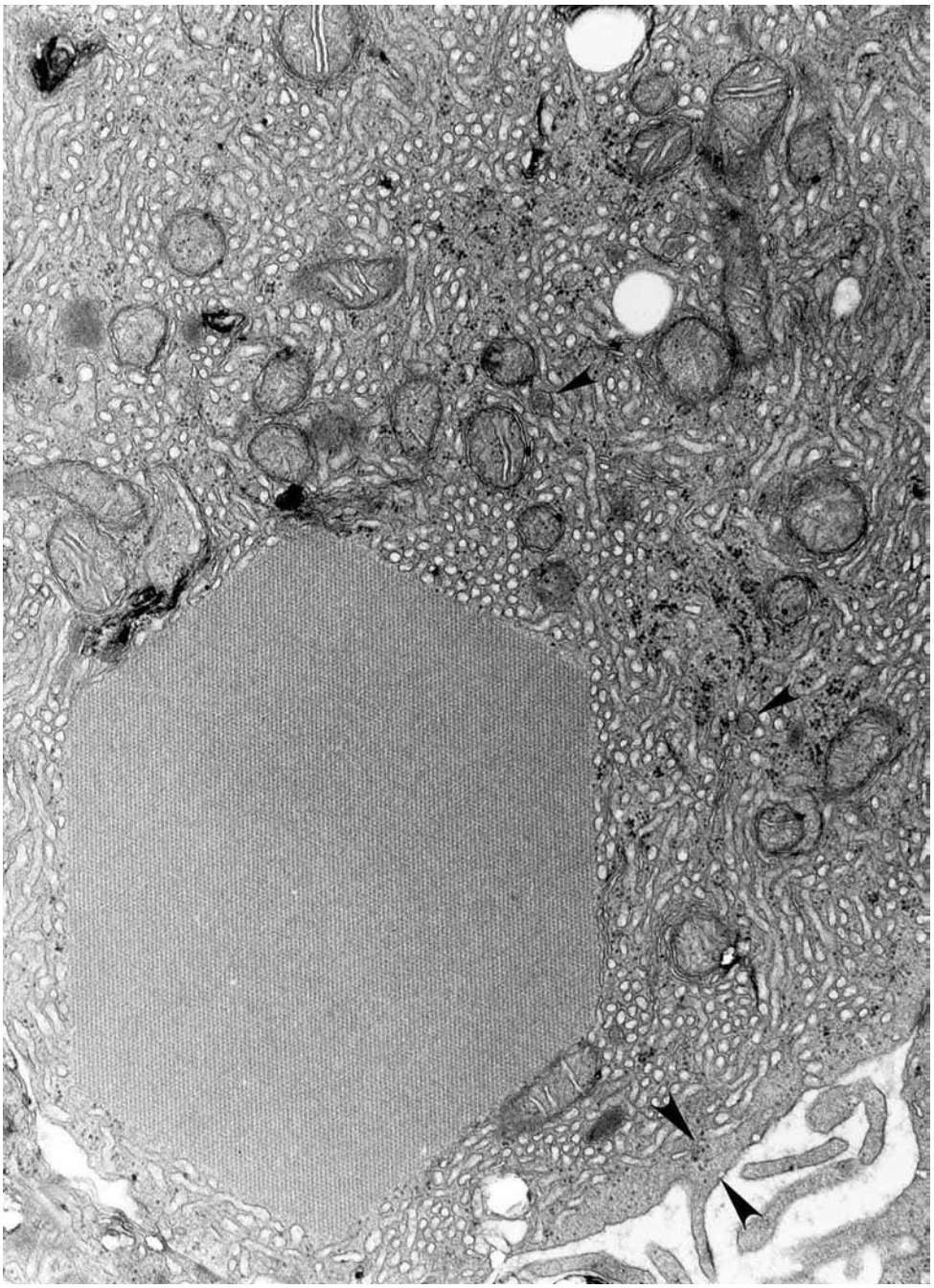


Fig. 3. This low-magnification micrograph vividly demonstrates the extensive network of anastamosing tubules of smooth endoplasmic reticulum throughout the cytoplasm. Profiles of mitochondria are numerous. Although the profiles are mainly circular in this TEM section, mitochondria are linear with branching. Microperoxisomes (small arrowheads) are dispersed within the maze of smooth endoplasmic reticulum. The ectoplasmic region is typified by a network of thin filaments beneath the cell membrane (between large arrowheads). Microvilli are also frequent. A Reinke crystal is obvious ×25,000.

is a feature of the mature neonatal Leydig cells as well as the ALCs. The LA form of the cristae appears unique to steroid-producing cells as a survey of approx three dozen cell types from all four major tissue types did not find any evidence of the LA in nonsteroid cells *(47)*.

The functional significance of the LA is unknown. Why is such a subcomponent of the cristae present? The morphology strongly suggests that these cristae membranes are not involved in ATP production because the narrow gap between the adjacent cristae does not

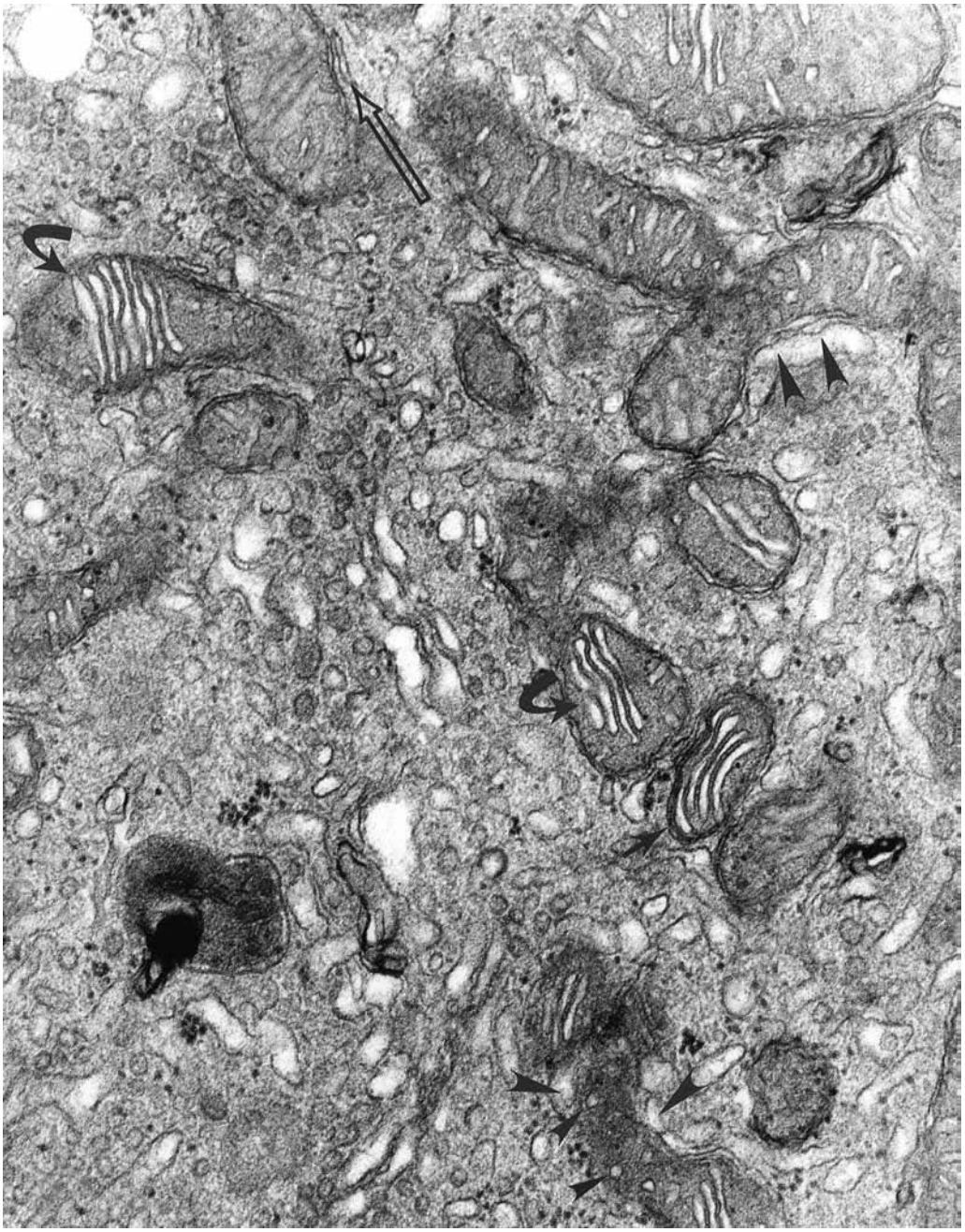


Fig. 4. Mitochondria of human Leydig cells contain tubular, lamellar, and pleomorphic cristae. The complexity is difficult to appreciate in a single TEM section. The new paradigm of mitochondrial structure is the "crista junction," the opening of the crista membrane to the inner boundary membrane (IBM). These roughly circular attachments, of approx 30 nm diameter, can be seen in TEM sections where the IBM is cut tangentially. Such tangential cuts are distinctly more electron dense than the mitochondrial matrix. Two crista junctions are indicated at the bottom of the figure by small arrowheads. An intriguing component of the cristae in these steroid-producing cells is cristae membranes which are very closely apposed, with a gap of approx 4 nm between the membranes. These are referred to as LA *(46–48)*. Two are shown by curved arrows, whereas a third is closely apposed to the IBM, suggesting membrane fusion (arrow). Mitochondria are in close association with the smooth endoplasmic reticulum (large arrowheads), as well as with the flattened MAM (open arrow). A Golgi region exhibiting numerous vesicles runs north to south in the micrograph ×58,500. (From ref. *46*. Reproduced with permission from John Wiley & Sons, Inc.)

allow matrix space for the F1 complex of ATP synthase *(47,48)*. Perhaps the LA is a temporary morphology representing a specific step during steroidogenesis. Of possible significance, examples of the LA are found with one lamella in close apposition to the IBM (Fig. 4 and color plate) (*see* previous work, ref. *46*), perhaps indicating sites of membrane fusion.

Although it is commonly stated that following conversion of cholesterol to pregnenolone all other enzymes of steroid synthesis are within the membrane of SER, there is evidence that a fraction of 3-β-hydroxysteroid dehydrogenase resides within the mitochondria in rat Leydig cells *(29)*. Also of interest, the δ4 pathway, predominant in many species including rat, would sequentially involve 3-β-hydroxysteroid dehydrogenase following pregnenolone formation, but the δ5 pathway, dominant in human, would not. This could partially explain subtle structural differences among species in cristae structure.

A critical, and yet unresolved, question is the mechanism of pregnenolone transfer from the cristae membranes to the SER. Morphological evidence does suggest a close apposition of the SER with the OMM (Fig. 4). Future studies are needed to elucidate possible protein translocons and fusion mechanisms between these membranes, as has been documented in the case of the mitochondrial membranes (OMM and IBM). Testosterone, the end product of this pathway, is apparently "secreted" from the cell via diffusion as there exists no morphological evidence of secretory granules.

The Golgi, Endocytic Pathway, and Delivery of Cholesterol to the Mitochondria

The delivery of cholesterol to the cristae (often the terminology IMM is used however, in this chapter the internal membranes of the mitochondria are separated into the IBM and the cristae) is the rate-limiting step in steroidogenesis *(49,50)*. Cholesterol can be synthesized *de novo*, mobilized from stored cholesterol esters (lipid droplets) or obtained through the uptake of low density lipoprotein (LDL) *(51)*. In human Leydig cells the morphology suggests that the endocytic/lysosome mechanism of cholesterol delivery is significant (*see* Fig. 1 for summary). Coated pits and CVs, the uptake mechanism of LDL, are evident at the cell surface and early endosomes (EE) and late endosomes (LE) are present in most sections. These EE and LE are also frequently referred to as pale and dense MVB. Figure 5 well demonstrates this variation. The pathway of endocytosed material through endosomes to the lysosmal and Golgi compartments has been well demonstrated by the study of Hermo et al. *(53)*. More specifically, the uptake of cholesterol and subsequent movement through the endosomal pathway to lysosomes and Golgi has been shown by Paavola et al. *(52)*.

The heterogeneity of the secondary lysosomes in human Leydig cells is striking (Figs. 2, 5, and 6). Electron lucent, crystaloid components are common, as are dense granular aggregations. Lipofuscin, the common term for lysosomal morphology when the organelles appear very heterogeneous, will be included under the terminology secondary lysosome in this chapter. Of interest and unknown significance, small angular electron lucent inclusions within lysosomes are a distinctive feature of the neonatal Leydig cells (Fig. 5), but not ALCs. Noteworthy is the close association of these secondary lysosomes with mitochondria (Figs. 2, 5, and 6) as it has been demonstrated that cholesterol within the endosomal/lysosomal pathway can be directly transferred to mitochondria *(54)*. Pelletier et al. *(55)* have, through EM cytochemistry, shown the content of cholesterol enriched membranes within Leydig cell lysosomes, as well as a subset of the cristae.

Myelin figures, a term often used for layered profiles of electron dense membranes, have been reported in the vicinity of mitochondria and lipid droplets in a number of studies of Leydig cells. Such compacted membranes are often present in close apposition with mitochondria in human Leydig cells (Fig. 8). Of significance, free cholesterol has been found to be stored in membranes *(55)*.

Human Leydig cells contain well-developed Golgi elements (Fig. 7) exhibiting the layered cisternae, fenestrated cisternae, and tubular and vesicular components, which reflect the function of the Golgi as such an intricate membrane transfer center. Golgi complexity in a variety of cells has been demonstrated by a number of excellent studies *(56–59)*. In Leydig cells, the Golgi of course is also involved with the production of lysosomes and likely involved with intracellular cholesterol transport, and membrane recycling, as shown in the adrenal cortex *(60)*.

Lipid droplets (stored cholesterol esters) are present in human Leydig cells and thus, are another source of cholesterol for steroidogenesis (Figs. 2 and 8) *(61)*. Subtle variation in morphology is found along the peripheral border. Often lipid droplets have a hazy periphery, whereas others exhibit an enveloping membrane; perhaps best termed the lipid-associated membrane (LAM)

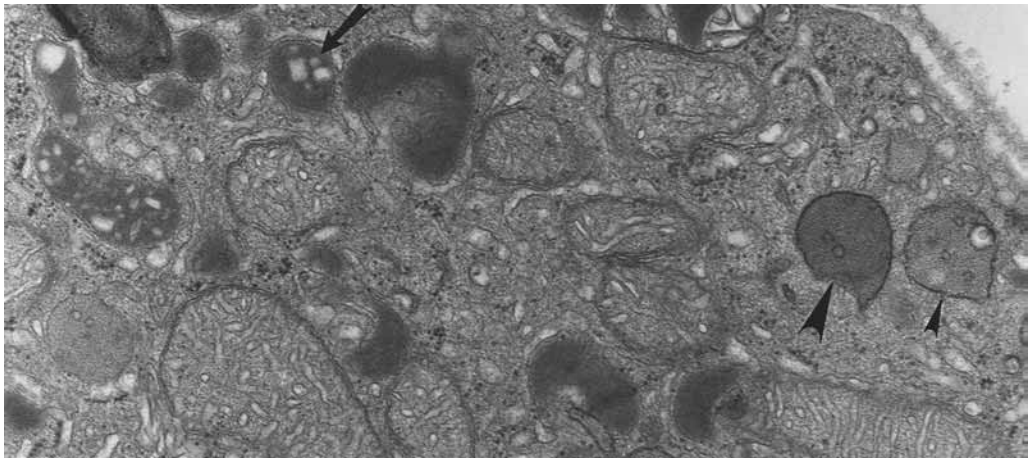


Fig. 5. Mature Leydig cell of the neonatal developmental phase. A region of cytoplasm exhibiting endosomes (also referred to as MVB) and lysosomes. As endosomes "mature" they gain electron density. An early endosome is indicated by the small arrowhead and a late endosome by the large arrowhead. Lysosomes are very heterogeneous in Leydig cells. Angular, lucent substructures are seen in these (arrow) ×28,000.

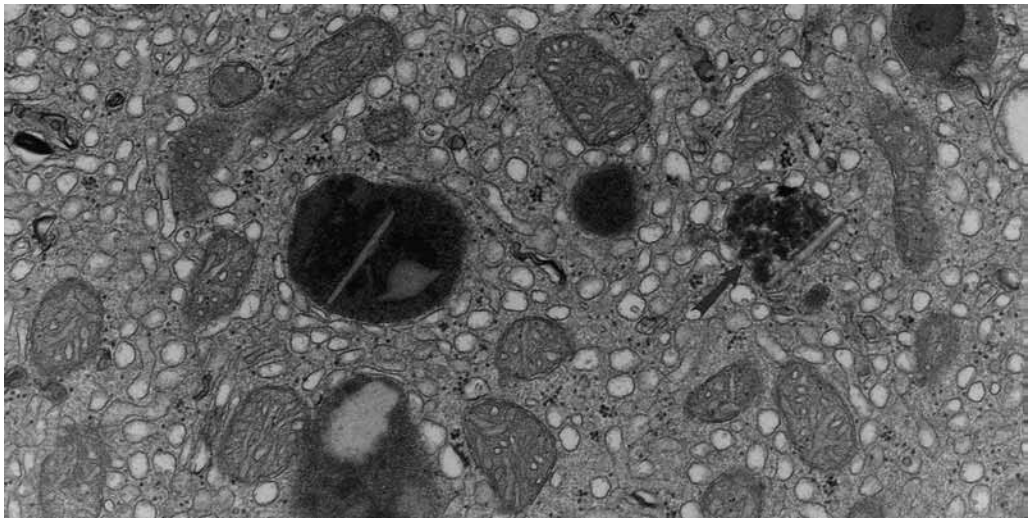


Fig. 6. Lysosomal variation is demonstrated. Often secondary lysosomes (the terminology lipofuscin is included with secondary lysosome in this chapter) exhibit a blotchy, electron dense material (arrow) ×33,000.

(Fig. 9). It is possible this is a cholesterol-enriched membrane on the surface of the stored cholesterol esters because cholesterol is stored within membranes *(55)*. Furthermore, free cholesterol has been localized on the surface of lipid droplets following LH stimulation in rat Leydig cells *(62)*.

The presence of a "cap" on one pole of the lipid droplet which extends to varying degree across the sphere is common and no doubt of significance in the transfer of cholesterol in or out. Often such a "lipid cap" has a central very dense region with a less electron dense homogeneous area tapering with distance over the lipid sphere (Fig. 8). Of note, the density and texture of this area is similar in consistency with the MP (Fig. 8). Other examples of a "lipid cap" are consistent with being lysosomal in origin.

The peroxisomes of Leydig cells from many species are typically found to be quite uniform in size and

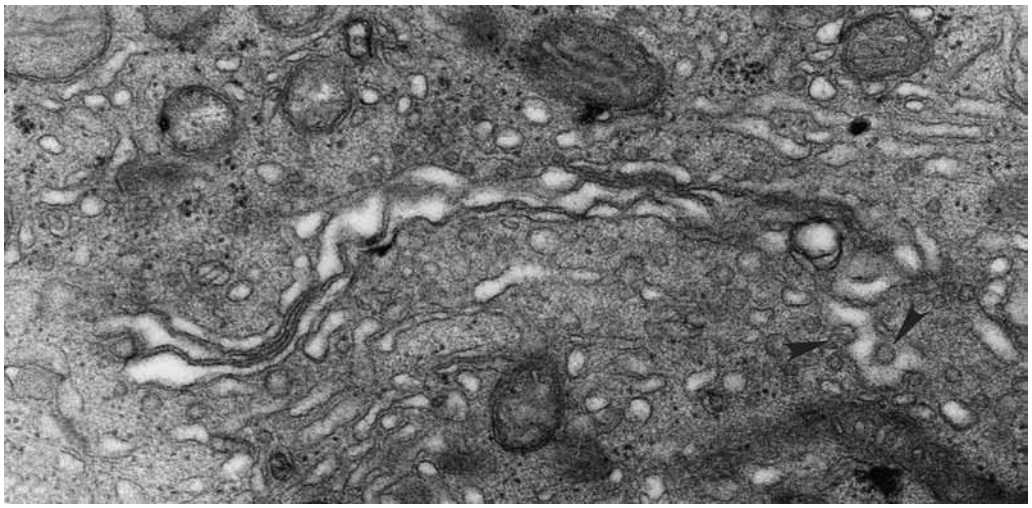


Fig. 7. The layered cisternae of a Golgi region is shown at higher magnification. A tangential cut reveals the fenestrations within the membrane (arrowheads) ×45,000.

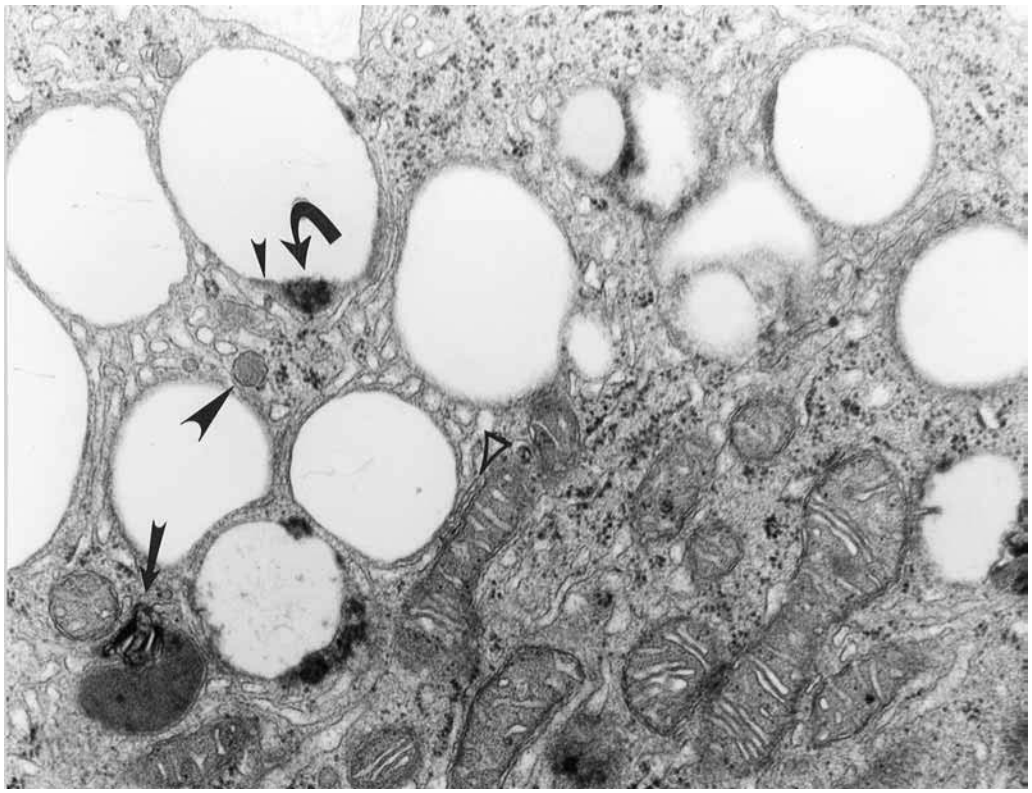


Fig. 8. Lipid droplets (stored cholesterol ester) are typically found in focal regions of the cytoplasm. Often a "lipid cap" is present. The morphology of these "caps" varies. The example shown (curved arrow) exhibits a central dense mass with less dense content as the cap extends over the sphere of the lipid droplet (small arrowhead). Of possible significance, the density and particulate content of this region of the cap is consistent with the morphology of a microperoxisome (large arrowhead). Layered, electron dense membranes referred to as "myelin figures" are often adjacent to lysosomes and mitochondria (arrow). A thin mitochondrial-associated membrane is indicated between a mitochondrion and a lipid droplet (open arrowhead) ×39,000.



Fig. 9. Lipid-associated membranes, occasional findings, are closely apposed to the surface of the lipid droplet (between the arrowheads) ×35,000.

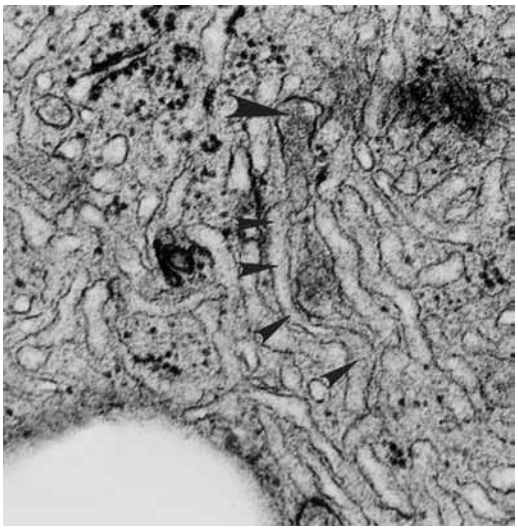


Fig. 10. Evidence of a continuity of microperoxisome (large arrowhead) with the smooth endoplasmic reticulum (small arrowheads) is present in human Leydig cells ×55,000.

significantly smaller than the peroxisomes of many cell types *(2–6,63–67)*, liver and kidney being notable examples *(67)*. Hence, the nomenclature MP. These MPs, generally 0.1–0.2 µm in diameter are found scattered throughout the SER (Fig. 3) and are known to contain sterol carrier protein-2, which is involved with mobilization of cholesterol *(62)*. Peroxisomes can also synthesize cholesterol *(68)*. Although not previously reported in the literature on human Leydig cells, more linear and dumbell forms are seen. This is consistent with existing studies on mouse *(69)* and rat *(70)* Leydig cells. Also, a continuity of a MP with the SER is occasionally observed (Fig. 10). This is a novel finding for human Leydig cells and has only been described previously in a single study of mouse Leydig cells *(71)* and also in the adrenal cortex *(49)*. Peroxisomes of a larger size, although relatively rare, are also present.

The delivery of cholesterol to the inner membranes is dependent on two proteins, steroid acute regulatory protein, and peripheral-type benzodiazepine receptor *(51,72)*. This involves membrane fusion. Mitochondrial associated membranes (MAM) are likely involved in this process. These membranes are flattened and distinctly different from the bulk of the SER (Fig. 4). Morphological contact points, sites of fusion of the OMM and IBM, have been shown to be involved in the transfer of cholesterol *(73)*. Although typically described as being rather discrete regions of membrane contact, "contact sites" between extensive areas of the OMM and IBM have been reported from this lab *(46)*. Evidence of fusion of peripheral membrane of the LA with the IBM was also presented *see* ref. *46* (Fig. 4). This suggests a mechanism of transfer of cholesterol to the cristae membrane compartment. MAM have been demonstrated to be specialized regions of the ER involved in translocation of phosphatidlserine into mitochondria for the production of phosphatidlethanolamine *(74,75)*.

Thus, the earlier described morphologies of the surface membranes on lipid droplets (LAM), the intimate association of lysosomes and peroxisomes with lipid droplets, the close association of such "activated" lipid droplets with MAM and mitochondria, the proximity of secondary lysosomes to mitochondria and the contact regions between the OMM and IBM (as well as cristae membranes of the peripheral membranes of the LA form of the cristae) all suggest extensive membrane fusion being important in cholesterol transfer through the cell and delivery to the cristae compartment.

Other Ultrastructural Features of the Human Leydig Cell

Ribosomes are scattered throughout the cytoplasm. Similarly, small elements of rough endoplasmic reticulum (RER) are widely dispersed, often adjacent to mitochondria. These small cisternae of RER are very often in continuity with elements of the SER. RER, when present in layered cisternae, is typically located toward the periphery of the cell (Fig. 8). It may be that a functional difference exists between the organized peripheral cisternae of RER and the small elements of

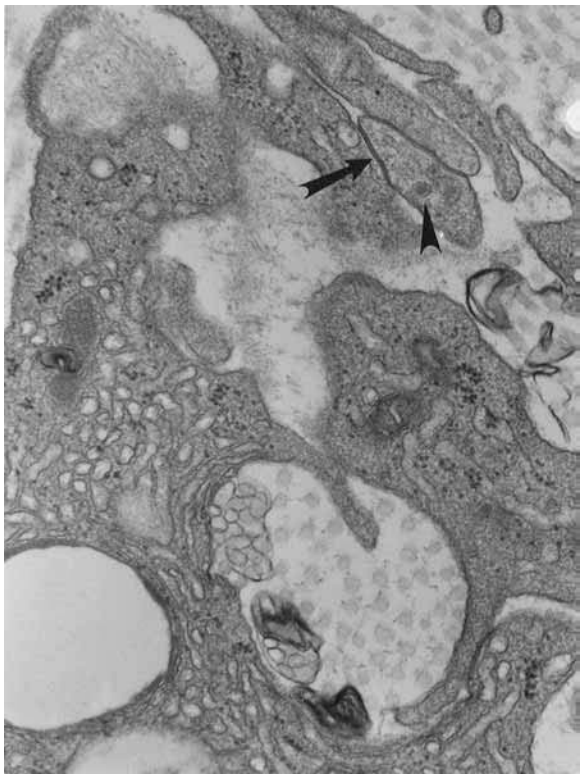


Fig. 11. Cell processes are common in mature Leydig cells and often are linked to other Leydig cells through gap junctions (arrow). Dense core granules are a consistent finding in cell processes (arrowhead) ×33,000.

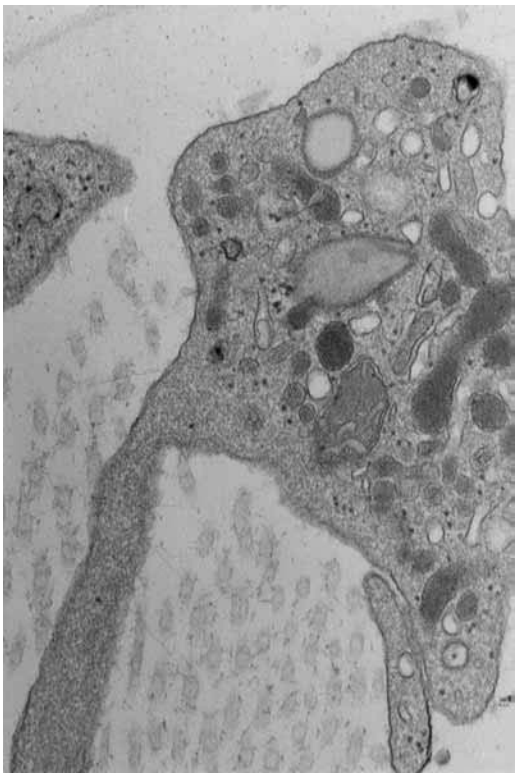


Fig. 12. Human Leydig cells occasionally exhibit expanded processes with an impressive amount of dense core granules ×39,000.

RER, which are so intrusive throughout the cell. Perhaps the peripheral stacked cisternae of RER are preferentially involved with paracrine function.

A distinguishing feature of the adult, but not fetal, or neonatal, human Leydig cell is the Reinke crystal *(76)* (Fig. 3). The substructure of this striking inclusion has been studied in detail *(4,77,78)*. Oddly, no function is known. It has been reported that these inclusions increase with age *(79)*. Although the Reinke crystal has been thought to be unique to human Leydig cells an inclusion with a similar substructure has been described in marmoset Leydig cells *(80)*. A variety of "paracrystalline" inclusions have also been recorded *(77,78)*.

The ectoplasmic region often exhibits a fine meshwork of thin filaments (Fig. 3). One function of the actin filaments is to provide a framework for microdomains in the cell membrane for caveoli and coated pits. CVs and noncoated endocytic vesicles are common beneath the cell membrane. Aside from microvilli, larger cell processes are common. Various dense core granules are often present in these larger processes, at times being surprisingly numerous (Figs. 11 and 12). This morphological feature seems "out of place" when focusing upon the steroid synthetic function of the cell; however, a number of studies have reported "neuronal" features to Leydig cells *(81–86)*. The presence of a variety of peptides, and so on, more typical of neurons is supported by this morphology.

Leydig cells are typically found in cell groups. Adherens junctions are common and more significantly, gap junctions are present between the cells (Fig. 11) *(2–7,87)*. For unknown reason, often gap junctions are between a cell process and the main cytoplasmic region of the second cell, giving a distinctive annular appearance *(6,87)*. Such annular gap junctions do become internalized (Fig. 3), perhaps as a mechanism of control of gap junction membrane. The full significance of gap junctions between the Leydig cells is not known, but their presence suggests coordinated activity within a small cellular group.

Morphological evidence of an innervation, both direct and indirect, of human Leydig cells by the autonomic nervous system has been previously

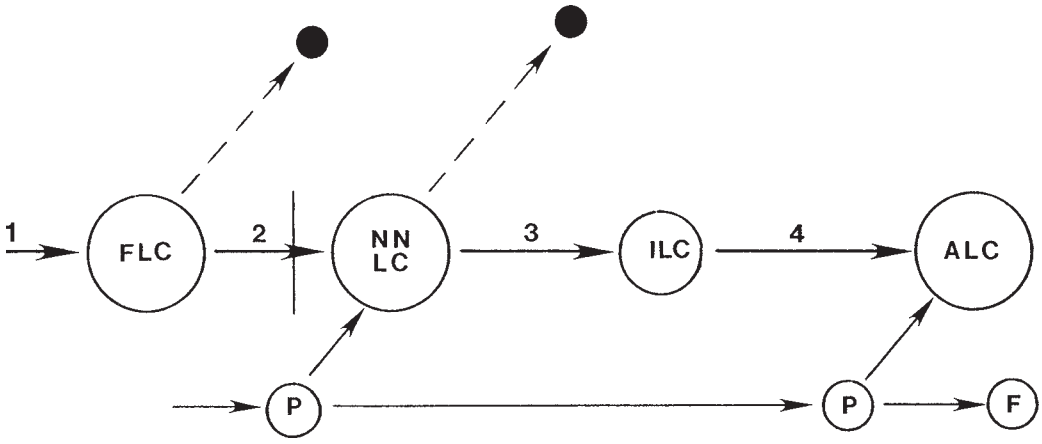


Fig. 13. Diagram of the triphasic nature of Leydig cell development in human. Although much remains unclear regarding the source of precursor cells and the relative and absolute numbers of cells involved, the evidence available supports this developmental sequence. The main horizontal axis indicates: (1) Development of the fetal Leydig cell population from undifferentiated precursors. (2) Regression of fetal Leydig cells, with subsequent maturation into mature neonatal Leydig cells (NN LC). Vertical line—Birth; A component of the FLC population degenerates; indicated by black circle. (3) Regression of NN LC population: Some cells regress to form the immature Leydig cell population (ILC) of childhood, whereas others degenerate (path to black circle). (4) Maturation of immature Leydig cells at puberty into a segment of the ALC population: this pubertal developmental phase also recruitment of precursor cells (P) from the interstitium and peritubular regions. Fibroblastic cells (F) of the adult interstitium are morphologically different from the primitive fibroblastic cells within the interstitium during childhood. (From ref *91*. Reproduced with permission from The Society of Endocrinology.)

reported from this lab *(88)*. The topic of Leydig cell control by neural elements has been reviewed by Mayerhofer *(89)* and will be covered by another chapter in this book.

LEYDIG CELL DEVELOPMENT IN HUMAN

In numerous mammalian species Leydig cell development is biphasic with fetal and adult populations. In contrast, the developmental history of human Leydig cells is triphasic, consisting of fetal, neonatal, and adult maturational phases (Fig. 13). The rationale for this triphasic developmental scheme was presented in detail in previous articles *(87,90,91)*.

The ultrastructure of human fetal Leydig cells (FLC) has been reported by a number of investigators *(92–95)*. In general, the morphology of the FLC is consistent with that of the ALC with the exception that no Reinke crystals are present. The proliferation of fetal cells active with the enzymes for steroid synthesis has been demonstrated by immunocytochemistry as well *(96)*. Following the peak of testosterone production from 14 to 18 wk of fetal life there is a period of involution with cell degeneration and regression *(92–95)*. Haider has reported a dramatic decrease in Leydig cell number from 19 to 23 wk *(13,97)*, which correlates with the decline in testosterone production. At the end of gestation few Leydig cells are present in the interstitium *(98)* with most cells being mesenchymal.

The hypothalamic–pituitary–testicular axis is again activated neonatally *(99–101)*, with a peak in testosterone production at 2–3 mo *(101,102)*. Mature Leydig cells have been demonstrated during this period by immunocytochemistry and electron microscopy *(87,90,95,103)*. Using a primate model it has been established that the development of the neonatal Leydig cell population is dependent upon an intact hypothalamic–pituitary axis and the release of gonadotrophic hormones *(104)*. The neonatal human Leydig cells share most ultrastructural features with the ALC, except for the absence of Reinke crystals. A more subtle difference is that secondary lysosomes in the neonatal cells often contain small, angular, lucent substructure (Fig. 5). Morphological evidence of cell regression, as well as some degeneration, is evident at 4 mo as previously reported *(87)*. Not mentioned in that article was the evidence of autophagy of cellular organelles in these cells. Also noteworthy, regressing cells often exhibit SER in the form of fenestrated cisternae, a feature not found in mature Leydig cells of

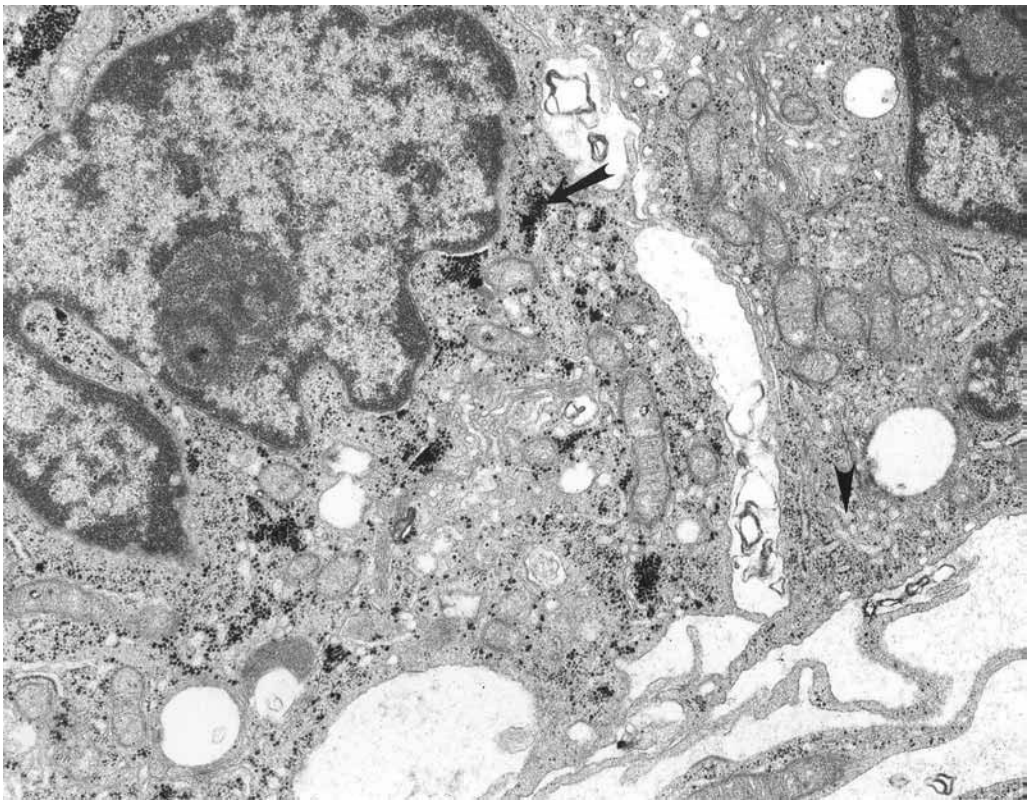


Fig. 14. Low-magnification micrograph of two immature Leydig cells (ILCs), characteristic of the testicular interstitium during childhood. These cells have irregular nuclei and modest heterochromatin. The cytoplasm of the (ILCs) contains well developed Golgi, mitochondria, occasional lysosomes, and lipid, and, the most impressively, tubular elements of smooth endoplasmic reticulum (arrowhead). Scattered clumps of glycogen (arrow) are a consistent feature and a distinct difference from the mature Leydig cells which do not exhibit glycogen ×18,000.

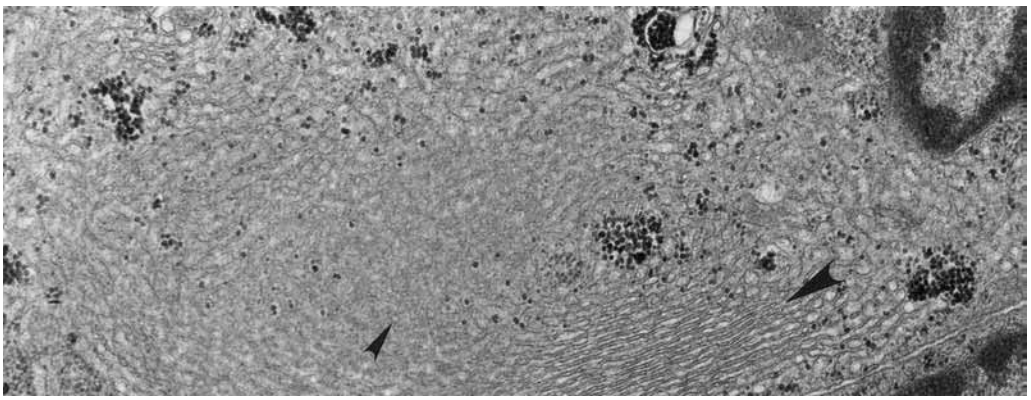


Fig. 15. Immature Leydig cell (ILC) of childhood. This micrograph exhibits a rather extensive area of layered, fenestrated lamellae of smooth endoplasmic reticulum. The large arrowhead shows the lamellae in cross-section while the small arrowhead points to fenestrations in the membranes as seen in "surface view." This common feature of the ILC is not present in the mature ALC ×33,000.

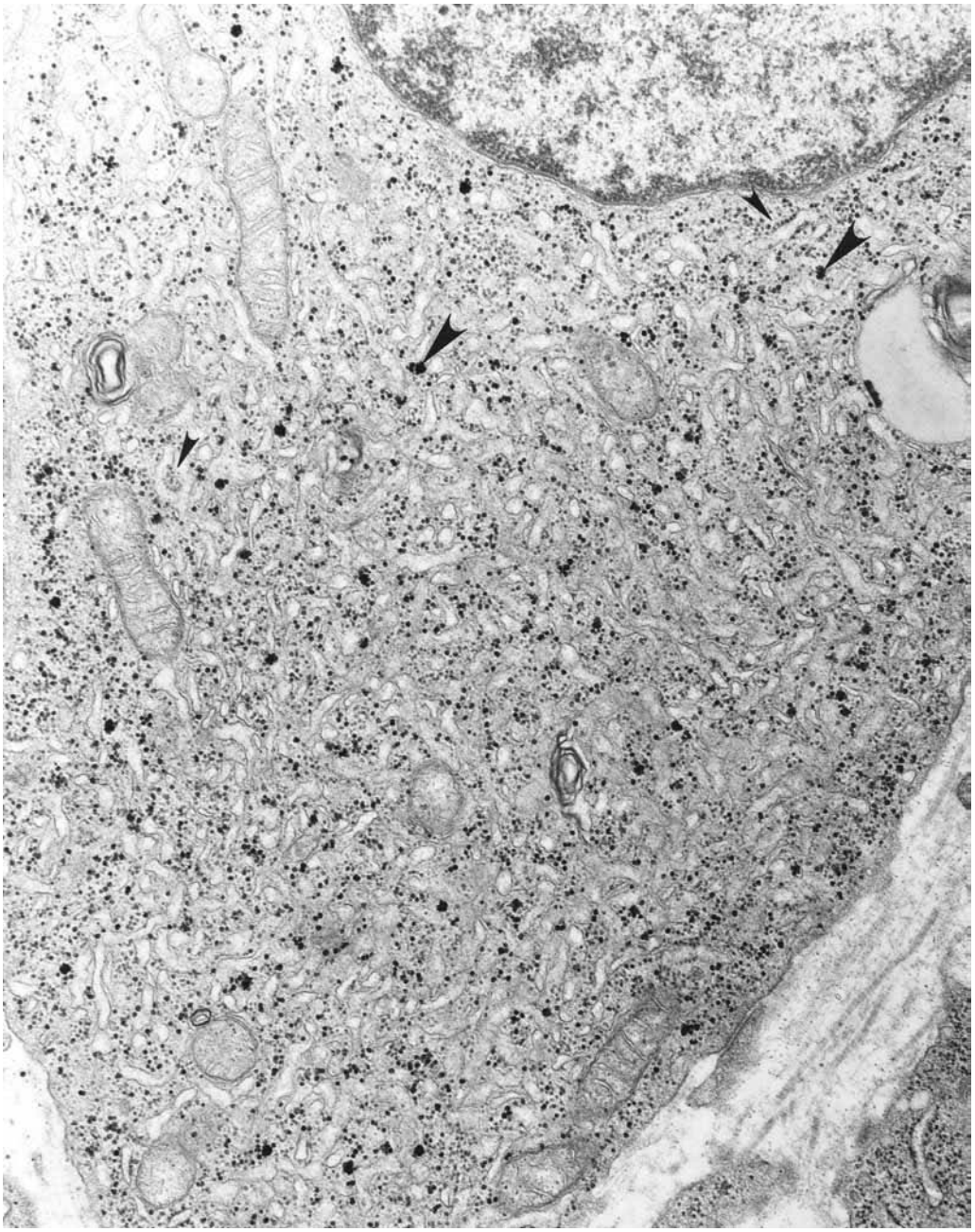
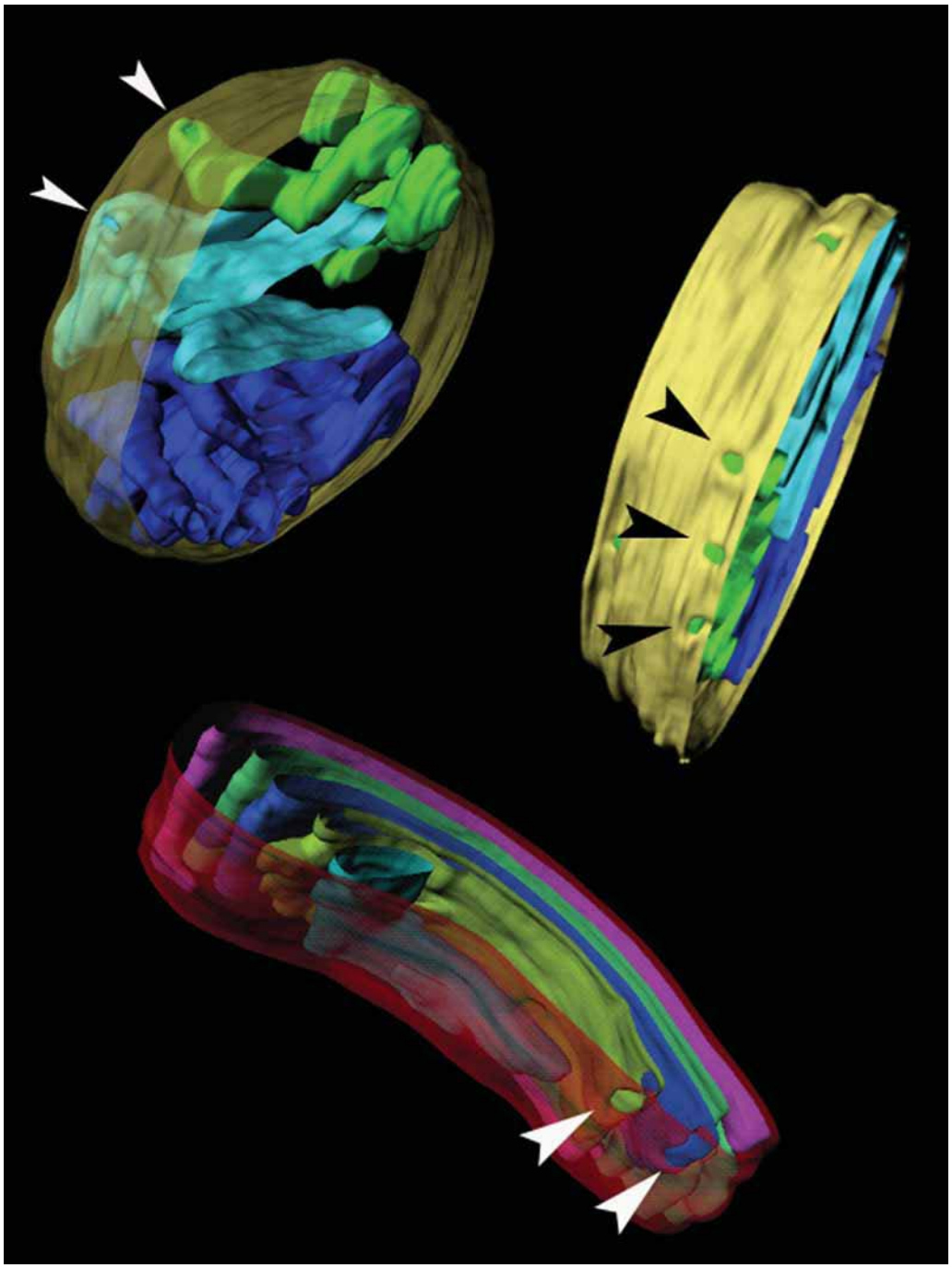


Fig. 16. Late prepubertal period: Leydig cells are present which appear to be in transition from the ILC to the mature ALC. Note the spherical contour of the nucleus and the extensive network of tubular smooth endoplasmic reticulum. Glycogen is present but distributed more homogeneously throughout the cytoplasm than typical of the ILC (e.g., two large arrowheads). Ribosomes are very numerous, either as free ribosomes or those associated with the RER. Two examples are shown by small arrowheads. One near the nuclear envelope shows a small element of RER in cross-section, whereas mid left in the figure appears to be a surface view of the rosette of ribosomes on the RER membrane ×33,000.

Color plate. Electron microscopic tomography allows for 3D reconstructions of segments of mitochondria. The top left reconstruction is 0.15 μm thick and thus, a small segment of the entire mitochondrion. The outer mitochondrial membrane is not shown. The inner boundary membrane (IBM) is transparent and the pleomorphic nature of the cristae is evident. One tubular crista (green) is present and the circular, approx 30 nm diameter attachment (opening) to the IBM is indicated by an arrowhead. Such attachments are referred to as crista junctions. Two interconnecting lamellar cristae (blue) also connect to the IBM through



Color plate. *(Continued)* a crista junction (arrowhead). The highly pleomorphic purple crista does not have a connection to the IBM in this view of the model. Top right: This is the same reconstruction; however, the IBM is opaque (the outer mitochondrial membrane is again not shown). The model has been rotated to indicate five crista junctions from different cristae (three are indicated by arrowheads). Bottom reconstruction is a 0.20 μ thick section of a mitochondrion which is rather elongate in profile. This contains the LA form of the cristae. Five lamellar cristae membranes are very closely apposed to each other and represented by different colors. The lamella, which is pink, is also closely apposed to the IBM (transparent and red). The significance of the LA form of the cristae is unknown but intriguing. These cristae membranes are too close to allow space for the F1 complex of ATP synthase on the matrix side of the membranes. Although this view appears to show these five lamellae as separate cristae, they all interconnect and thus, form a continuous internal membrane system. The yellow and dark blue cristae open to the IBM through crista junctions (arrowheads).

any developmental phase. Another intriguing finding in a subset of regressing neonatal Leydig cells is the general paucity of mitochondrial cristae with the exception of examples of the LA.

The immature Leydig cells (ILC) of childhood are very likely regressed remnants of the neonatal Leydig cell population. The morphology of the ILC has been described and their relative numbers found to remain constant from the age of 3–8 yr *(105)*. These cells exhibit an irregular nucleus, and are very "geared down" regarding the organelles of steroidogenesis, but can quickly respond to hormonal stimulation *(106–108)*. Although the anastamosing tubular form of SER is common, cisternal SER is also a consistent ultrastructural feature (Figs. 14 and 15). A unique cytoplasmic feature is the presence of glycogen, typically found in scattered clumps (Fig. 14). This suggests metabolic pathways which are not utilized by the mature Leydig cell.

The majority of the cells comprising the testicular interstitium during childhood exhibit no evidence of a capacity for steroid synthesis and have been described as primitive fibroblastic cells because of their undifferentiated morphology *(105)*. Likewise, the cells surrounding the seminferous tubules (lamina propria) are not yet differentiated into myoid cells, but are quite undifferentiated.

Cell morphologies consistent with being transitional from ILC to ALC are observed in the older prepubertal specimens (age 9–11 yr). These cells are larger than immature Leydig cells, exhibit a more spherical nucleus and a strikingly "geared up" cytoplasm for steroidogenesis with extensive profiles of tubular SER (Fig. 16). Rapid changes in biosynthesis are reflected by the high numbers of free ribosomes and ribosomes associated with small elements of the endoplasmic reticulum. Also noteworthy, glycogen is not as localized in clumps but is often widely scattered throughout the cytoplasm. As the relative numbers of ALCs increase during puberty there is a concomitant decrease in the number of primitive fibroblastic cells (F. P. Prince, unpublished observation). Thus, the pubertal development of the ALC population likely is a combination of maturation of the immature Leydig cells and recruitment of precursors from the interstitium and peritubular region.

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Specimens and Methodology: the testicular specimens are from archival diagnostic biopsies taken at Children's Hospital, Columbus, Ohio for the purpose of establishing the presence or absence of leukemic cells. Cases negative for leukemia have been used in a series of studies at the Department of Biological Sciences, Plymouth State University over the years utilizing a Hitachi HU-11E TEM and more recently a Philips 410LS TEM. Thanks also to Dr. William Newton for the continued use of this material. Detailed methodology can be found in the earlier articles *(46–48,87,88,90,91,105)*.

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6 Dynamics of Leydig Cell Regeneration After EDS

A Model for Postnatal Leydig Cell Development

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SUMMARY

Synthesis and secretion of androgens is the most important function of Leydig cells in the testis. The levels of androgens produced by these cells not only depend on their capacity to produce these steroids, but also on the number of Leydig cells present in the testis. Therefore, it is essential to understand how the formation of the Leydig cell population is regulated and to identify the factors, which play a role in this developmental process. However, the initial studies to investigate the regulation of Leydig cell development were not undertaken in the (pre)pubertal testis but in the adult testis. With the identification of ethane-1,2-dimethyl sulphonate (EDS) as a specific Leydig cell toxicant a large number of studies were initiated. The latter was because of the fact that following EDS administration a completely new Leydig cell population was formed. This chapter summarizes more than 20 yr of research on Leydig cell development in the adult testis using EDS as a model. The sensitivity of Leydig cells in different species for the cytotoxic action of EDS is discussed as well as the possible mechanism of action of this cytotoxic compound. A comparison is made between Leydig cell development in the (pre)pubertal testis and the adult testis during the regeneration process following EDS administration. Specific emphasis is paid to the regulatory role of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) as well as other systemic and locally produced factors, such as thyroid hormone, insulin-like growth factor (IGF)-1, and transforming growth factors (TGF)-α and TGF-β, in this developmental process. It is concluded that there appear to be many similarities and hardly any discrepancies in the regulation of the development of precursor cells into mature adult-type Leydig cells during (pre)puberty and in the adult rat following EDS administration.

In the perinatal period when the stem cells become committed to lineage-specific differentiation, there are also differentiated Leydig cells present in the interstitium, namely, the fetal-type Leydig cells, which could influence the development of the adult-type Leydig cell population. Moreover, the intratesticular microenvironment of the (pre)pubertal testis is presumably rich in growth and differentiation inducing factors, whereas not only adult-type Leydig cells are developing but also other somatic cells are undergoing growth and differentiation. In contrast, following EDS administration in the adult animal, all differentiated Leydig cells are eliminated; the only undifferentiated cells left are presumably the stem cells/precursor cells. Taking into account the aforementioned, although EDS is a toxic compound which might influence the testicular microenvironment, the similarities between adult-type Leydig cell development in the (pre)pubertal testis and Leydig cell regeneration after EDS, make it tempting to speculate that the EDS-treated adult rat is better model for the study of the regulation of adult-type Leydig cell development than the (pre)pubertal testis. It is easier to follow cellular differentiation and ontogeny when no other mature cells are around.

Key Words: Adult-type Leydig cell development; EDS; Leydig cell regeneration; LH; FSH; LH receptor; steroidogenic enzyme.

INTRODUCTION

The most important function of Leydig cells in the (pre)pubertal and adult testis is the synthesis and secretion of steroids, 5α-reduced androgens and testosterone, which are essential for the progression of spermatogenesis. In the 1970s and 1980s, much emphasis was put on the regulation of androgen synthesis by the Leydig cells. However, the levels of androgens produced by the testis not only depend on the capacity of these cells to produce steroids, but also on the number of Leydig cells present in the interstitium.

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Therefore, it is essential to understand how the Leydig cell population in the adult testis is formed, and which factors play a role in the regulation of this developmental process. However, the initial studies concerning the regulation of Leydig cell development in the testis were not undertaken in the (pre)pubertal testis but in the adult testis. In the early 1980s, it became apparent that it was possible to selectively destroy the complete Leydig cell population in the adult rat testis within 3 d by treating the animals with a single dose of the alkylating agent EDS. This process was followed by a complete regeneration of the original Leydig cell population within a few weeks *(1–3)*. In this chapter a comparison will be made between the regeneration of the Leydig cell population after EDS administration and the development of the adult-type Leydig cell population in the (pre)pubertal testis. Although, it has been demonstrated in the past 10 yr that EDS also acts as a Leydig cell toxicant in other species, such as mouse *(4–7)*, guinea pig *(7)*, hamster *(7–9)*, monkey *(10)*, rabbit *(11)*, frog *(12,13)*, and lizard *(14)*, the main emphasis in this chapter will be on the rat. Evidence will be presented that Leydig cell regeneration in the adult testis following EDS, and the formation of the adult-type Leydig cell population in the (pre)pubertal testis develop along the same lineage.

Gonadotropins and Leydig Cell Development

Generally, in the rodent testis it has been accepted that two waves of proliferation and differentiation can be discerned during the development of the Leydig cell population. The first wave takes place during the fetal life independent of LH *(15–18)*, whereas the second wave occurs during the (pre)pubertal period and is largely dependent on LH *(19–22)*. After birth the population of fetal-type Leydig cells decreases in size, although some fetal-type cells persist even in the adult testis *(23)*. The second generation of Leydig cells, the so-called adult-type Leydig cells, develops through several steps of proliferation and differentiation *(15,20,21)*. Stem cells which are platelet-derived growth factor (PDGF) receptor-α-positive, but do not express steroidogenic enzymes or LH receptors, are believed to differentiate into mesenchymal-like precursor cells *(24)*. These precursor cells also do not express steroidogenic enzymes but do acquire LH receptors before they differentiate into 3β-(HSD) hydroxysteroid dehydrogenase positive Leydig cell progenitors (Teerds et al., manuscript submitted). Subsequently, these cells undergo further proliferation and differentiation, and develop into immature adult-type Leydig cells, in which proliferation is limited. These immature Leydig cells undergo further differentiation into mature adult-type Leydig cells *(20,21,25)*. In the rat, this wave of adult-type Leydig cell development is initiated around day 14 after birth with the formation of the first Leydig cell progenitors. These cells undergo proliferation and differentiation and around day 35 postpartum become immature adult-type Leydig cells, which still undergo some mitotic divisions and finally, when proliferation ceases around the age of 60 d, become mature adult-type Leydig cells (Fig. 1; refs. *20,26,27)*. Each step in this developmental process is characterized by specific morphological aspects of the developing cells *(21,22,25,28)* and the expression of specific steroidogenic enzymes, such as 3β-HSD, 17β-HSD/17-ketosteroid reductase, and 5α-reductase *(29,30)*.

Although, some controversy exists whether mesenchymal-like Leydig cell precursors at the onset of differentiating into progenitor cells express LH receptors, in vivo studies have indicated that the conversion of precursor cells into Leydig cell progenitors and the subsequent proliferation and differentiation of the progenitors and immature adult-type Leydig cells into mature adult-type Leydig cells, is a gonadotropin-dependent process (refs. *21,31–37*; Teerds et al., manuscript submitted). Treatment of hypophysectomized immature rats with highly purified preparations of pituitary FSH or human recombinant FSH, had a stimulatory effect on the differentiation of mesenchymal-like precursor cells into Leydig cell progenitors, without affecting the proliferative capacity of these progenitors *(34–37)*.

Treatment with highly purified LH stimulated the differentiation of the precursors into progenitors as well as the proliferation of these newly formed Leydig cell progenitors *(36)*. Administration of the LH analog human chorionic gonadotropin (hCG) to (pre)pubertal boys whose testes were devoid of morphologically recognizable Leydig cells, also enhanced the differentiation of precursor cells into progenitors and immature adult-type Leydig cells *(32,33)*. When gonadotropin levels were very low or negligible, such as in hypogonadal mice *(38)*, or in case of disruption of LH signaling in LH receptor knockout mice *(39)*, adult-type Leydig cell development appeared to be severely affected. Taken together, these data indicate that the generation of a full complement of steroidogenically active mature adult-type Leydig cells is dependent on the presence of gonadotropins and functional gonadotropin receptors (Fig. 1). The mature adult-type Leydig cell population formed in this way is a stable population of cells that has long been presumed to be relatively insensitive to cytotoxic compounds and to irradiation *(40–43)*.

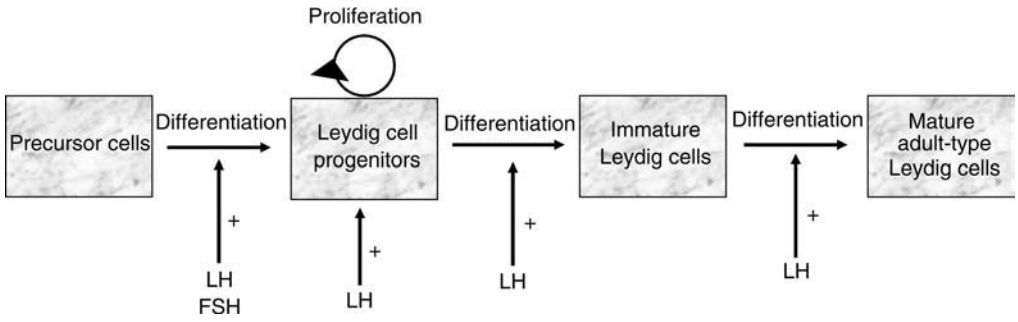


Fig. 1. A simple model for the development of Leydig cells. Mesenchymal-like precursor cells develop mainly under the influence of LH into steroid producing Leydig cell progenitors, although FSH can also slightly but significantly enhance this process. The proliferation (indicated by a circle with arrowhead) and differentiation of these cells into proliferating immature adult-type Leydig cells and subsequently into mature adult-type Leydig cells, is a process that is largely dependent on LH (modified from ref. *26*).

EDS: A Cytotoxic Compound That Affects Leydig Cells

Alkylation of DNA is the favored pathway by which many nonsteroidal antifertility agents exert their effect. The sensitivity of testicular cell types to these agents can differ considerably, depending on the cell type and age of the animal at the time of treatment. In general, rapidly proliferating cells, such as spermatogonia, are killed by most alkylating compounds *(44)*, whereas somatic cells with a limited proliferative capacity, such as Leydig cells and Sertoli cells, are relatively insensitive to these compounds *(40,43)*. One of these alkylating antifertility agents is EDS. This cytotoxic agent exerts a rather unusual pharmacological effect upon the testis of the rat and several other species, resulting in a temporary period of sterility for 2–8 wk after a single injection *(4,44–46)*. In contrast to the antifertility actions of homologous compounds, such as busulphan, ethane- and butane methyl sulphonate, the actions of EDS could not be attributed to a direct effect on germ cell proliferation, but appeared to be the result of impaired functioning of the androgen producing Leydig cells *(47–49)*. As a result of a single injection of EDS, testosterone production was inhibited within 24 h following administration *(49,50)*, only to return to pretreatment levels 3–7 wk after injection *(49)*. The observed decline in the weights of the testes and the accessory sex organs, seminal vesicles, and prostate, as well as the temporary impairment of fertility were believed to be the consequence of the decrease in testosterone production *(47–49)*.

Although, all these studies pointed to a direct cytotoxic effect of EDS on Leydig cell function, not before 1985, the basis for the temporary impairment was understood. Both Kerr *(1)* and Molenaar and colleagues *(2)* demonstrated at the same time that the observed decrease in plasma testosterone levels following EDS administration, was the result of the rapid elimination of the existing Leydig cell population (Fig. 2) through a process named programmed cell death or apoptosis *(51–55)*. Within 2–3 wk after a single dose of EDS, new Leydig cells could be observed within the testicular interstitium *(56,57)*, indicating that regeneration of the Leydig cell population was initiated (Fig. 2). Approximately 8–10 wk after EDS administration the Leydig cell population had returned to its original size (Fig. 2). These initial two studies formed the basis for a vast amount of other studies on different aspects of the process of Leydig cell destruction and subsequent regeneration in adult and aged animals *(3,7,58–79)*.

Most studies have focused on the effects of EDS on the regulation of the regeneration of the Leydig population after destruction. EDS has also been used as a tool to investigate the effects of testosterone depletion on the process of spermatogenesis. Some controversy exists as to whether EDS can exert a direct effect on spermatogenesis, independent from the effects of testosterone ablation. Sprando and colleagues *(80)* reported that in rats, in which, intratesticular testosterone levels were kept within the control range after EDS administration, spermatogenesis was affected in some seminiferous tubules. In the epididymis, EDS was shown to induce in a dose-dependent way, the formation of sperm granulomas in the caput epididymis, whereas in the corpus epididymis morphological alterations were observed. Moreover, sperm cells showed a progressive decrease in motility and velocity *(81)*. EDS treatment has also been reported to induce regression of the thymus in adult rats *(82)*. But in the adrenal cortex, EDS administration caused a marked decrease in the number of parenchymal cells in the zona fasciculate and also the formation of a layer of atrophic cells

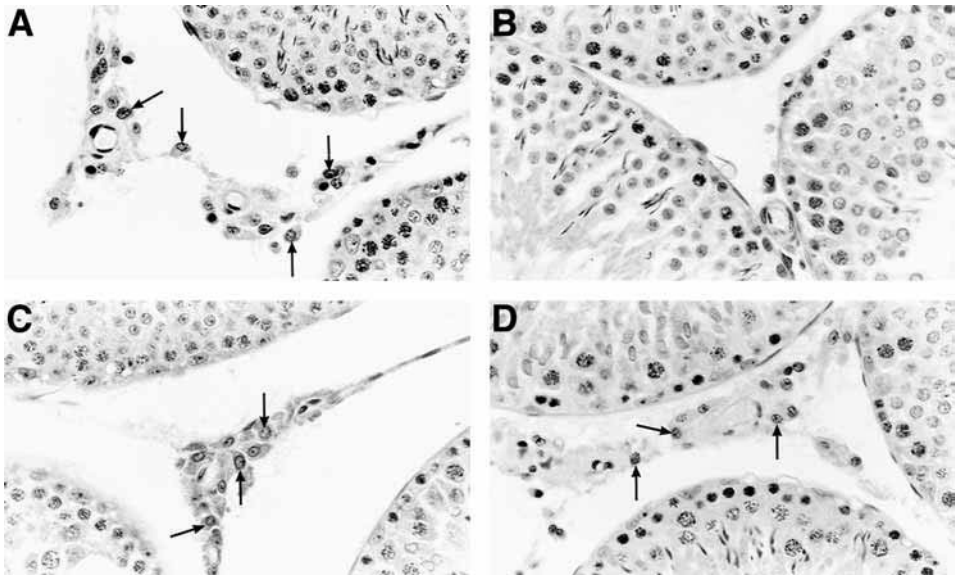


Fig. 2. Testicular histology of a control rat and animals killed 3, 21, and 54 d after EDS administration. Tissue was fixed in Bouins, embedded in JB4 resin and stained by the PAS reaction and Gill's hematoxylin. **(A)** Control testis, Leydig cells are indicated by arrows. **(B)** Testis 3 d after a single injection of EDS, the Leydig cells have by now disappeared from the interstitium. **(C)** Twenty one days after EDS administration, Leydig cell progenitors have appeared and are indicated by arrows. **(D)** Fifty-four days after EDS, the Leydig cell population has now completely regenerated.

at the border with the zona reticularis *(83)*. Although, these few studies suggest that EDS can exert direct cytotoxic effects on cell types other than Leydig cells, the general consensus is that EDS has limited toxicity in tissue other than the testis. Only when animals are exposed to EDS continuously for several days or weeks, more severe cytotoxic effects have been observed (e.g., ref. *84*). Therefore, the next paragraphs of this chapter will focus on the cytotoxic effects of EDS on Leydig cells and the regulation of the formation of a new adult-type Leydig cell population.

SENSITIVITY OF LEYDIG CELLS TO THE CYTOTOXIC EFFECTS OF EDS

Cytotoxicity of EDS Depends on the Developmental Stage of the Leydig Cells

Although, many proteins are alkylated following EDS administration *(85)*, the cytotoxic effects of EDS in the testis of the rat and several other species were highly selective: only mature adult-type Leydig cells were destroyed. Besides its effects on normal Leydig cells, EDS has also been shown to kill tumor Leydig cells in testicular neoplasms that developed during aging, as well as rat Leydig tumor cell lines *(85–87)*. The selectivity of the effects of EDS was further supported by the observation that EDS treatment of female rats did not result in destruction of ovarian thecal cells, which are the counterparts of Leydig cell *(84)*. Ovarian thecal cells are not terminally differentiated, in contrast to mature adult-type Leydig cells in the testis, indicating that EDS might only kill terminally differentiated steroidogenic cells. Indeed, when (pre)pubertal rats were treated with EDS at the age when Leydig cell progenitors are differentiating into immature adult-type Leydig cells, progenitors cells were not killed by the toxicant *(73,88,89)*. Although EDS was not cytotoxic to these cells, the function of the progenitors and immature adult-type Leydig cells was affected by EDS treatment. Both in vivo and in vitro studies reported that under these circumstances testosterone production was in general reduced *(73,84,88,90)*.

Taking into account that all Leydig cells in the adult testis are killed by EDS, a puzzling observation was made. When adult rats were treated with EDS on a weekly basis for 6 wk instead of receiving a single injection, serum testosterone levels returned quite unexpectedly to the normal control range between 4 and 6 wk after the first injection *(89)*. These results were comparable with the effects of a single dose of EDS *(58)* and suggested that; although, EDS was administered repeatedly, new testosterone producing Leydig cells were formed, that were insensitive to continued injections of EDS. Subsequent studies confirmed these findings and

furthermore demonstrated that the new Leydig cells formed after the first injection of EDS had different characteristics compared with mature Leydig cells in the adult testis *(63,89,91)*. The morphological appearance of the newly formed EDS-insensitive Leydig cells prompted some groups to suggest that these cells had fetal-type Leydig cell characteristics *(58–63)*. Whereas, other groups suggested that these newly formed EDS-insensitive Leydig cells showed similarities with Leydig cell progenitors, and immature adult-type Leydig cells in the (pre)pubertal testis *(89,91,92)*. The solution for this controversy came from experiments in which neonatal rats were treated with EDS *(93–96)*. In the testes of these animals fetal-type Leydig cells were the only steroid producing cell-type present *(15)*. These studies demonstrated that, like the mature Leydig cells in the adult testis, fetal-type Leydig cells in the neonatal testis were sensitive to the cytotoxic actions of EDS, suggesting that the regenerating Leydig cells after EDS injection develop from the same stem cell/precursor cell as the adult-type Leydig cells in the (pre)pubertal testis. Experiments, in which adult rats were treated with high doses of the LH analog hCG gave the final proof that indeed Leydig cell development in the adult testis takes place from the same stem cell/precursor cell as the development of Leydig cell progenitors/immature adult-type Leydig cells in the (pre)pubertal testis. In these experiments intact adult rats were treated with high doses of hCG for several days, before EDS administration *(97,98)*. Under these conditions, hCG stimulated the development of precursor cells into new Leydig cells *(68)*. Subsequent EDS treatment killed all mature adult-type Leydig cells, whereas the Leydig cells that had newly developed under the influence of hCG, appeared to be insensitive to the cytotoxic actions of EDS *(98)*.

Species Specificity and the Cytotoxic Effects of EDS

As indicated earlier, the initial studies describing the effects of EDS on testis morphology and function were carried out in the rat. At the time the selective cytotoxic effects of EDS on rat Leydig cells, the question was whether EDS could also be used in other species as a tool to kill Leydig cells and to study the effects of androgen ablation on spermatogenesis, as well as other endocrine and physiological aspects of testicular function.

The effects of EDS on testicular function show an intriguing species specificity, although in the last few years several new species have been added to the list of animals in which EDS exerts a cytotoxic effect on interstitial cell function. Initial studies reported that EDS had no effect on Leydig cell function and morphology in the mouse and Chinese hamster, not even when threefold higher doses and repeated injections were administered (*7,90*; Teerds and Janssen, unpublished data). More recent reports demonstrated that, depending on the dose and age of the animal at the time of treatment, EDS could exert negative effects on mouse Leydig cells as well. When a high dose of EDS was given to pregnant female mice, the Leydig cell population in their male off spring appeared to be reduced in size *(5)*, whereas in vitro studies showed that a dose 20-fold higher than what normally was used to induce rat Leydig cell apoptosis, caused apoptosis in a mouse Leydig cell line *(6)*. In the guinea pig, Siberian hamster and adult bonnet monkey *(Macaca radiata)* the effects of EDS administration were comparable with those in the rat *(7,9,10)*. Leydig cells in the boar testis were also sensitive to EDS; however, the minimal effective dose of 75 mg/kg body weight not only destroyed the Leydig cell population, but was also lethal to the adult boar. A lower dose of 50 mg/kg body weight did not affect the boars' health, but was not high enough to induce Leydig cell death (Teerds and Fentener van Vlissingen, unpublished results). In the Syrian hamster and the rabbit, EDS affected Leydig cell numbers, although the cells were less sensitive to the cytotoxic action of the drug than rat Leydig cells, because EDS did not kill all mature Leydig cells in these two species *(8,11)*. In contrast, EDS administration to goats did not have any effect on Leydig cell function or morphology but caused a severe disruption of the process of spermatogenesis *(99)*.

The effects of EDS on testicular function and morphology were also investigated in several lower vertebrates. In the frog, *Rana esculenta*, and the lizard, *Podarcis s. sicula Raf*, the effects of EDS were comparable with the rat. All Leydig cells were destroyed within 4 d after a single injection, followed by regeneration 28 d later *(12–14,100)*. Leydig cell regeneration in the frog appeared to occur even in hypophysectomized animals, independent of pituitary control *(101)*. Besides affecting the Leydig cells, EDS also inhibited spermatogonial mitosis in the frog *(102)*. In another lower vertebrate, the teleost administration of EDS during the nonbreeding season does not kill Leydig cells. On the contrary, Leydig cell androgen production is stimulated by a factor of 5–10-fold *(103)*. To this end, the teleost is the only species in which EDS has been found to stimulate Leydig cell function. An overview of the sensitivity of the different species to the cytotoxic actions of EDS is presented in Table 1.

Table 1
Overview of the Sensitivity of LC in Different Species for the Cytotoxic Actions of EDS

Species	*Sensitivity of LC to EDS*	*References*
Rat	+	For example, *1–3*
Mouse	+/–	*5,7,90*
Guinea pig	+	*7*
Chinese hamster	–	Teerds and Janssen (unpublished)
Siberian hamster	+	*9*
Syrian hamster	+/–	*8*
Bonnet monkey	+	*10*
Boar	+[a]	Teerds and Fentener van Vlissingen (unpublished)
Rabbit	+/–	*11*
Goat	–	*99*
Frog	+	*12,13,100,102*
Lizzard	+	*14*
Teleost	–	*103*

[a]The cytotoxic dose of 75 mg/kg BW not only destroyed the Leydig cells but also killed the boar. Lower doses did not have a cytotoxic effect on the Leydig cell population.

It is obvious from the above studies that the effects of EDS on Leydig cell function and morphology are species-dependent. As indicated earlier, most investigations have been carried out in rats, initially studying the process of Leydig cell depletion and regeneration. In the past 10 yr the focus of research has changed slightly as EDS has quite often been used as a tool to study the effects of androgen ablation on spermatogenesis as well as androgen regulated gene expression in the testis and accessory sex organs. Because of the complete absence of testicular androgen synthesis following EDS administration, this model might provide a better way to study androgen-regulated gene expression than gonadotropin-releasing hormone antagonist treatment, which suppresses LH release but not completely blocks testicular androgen synthesis.

Mechanism of Action of EDS

The first changes in Leydig cell morphology under the influence of EDS became apparent between 6 and 18 h after EDS administration, when the nuclear morphology of the cells acquired characteristics of apoptosis, such as chromatin condensation and at later stages nuclear fragmentation (ref. *104*; Fig. 3). Twenty four hours after EDS administration, most Leydig cells showed signs of apoptosis; by 72 h post-EDS all Leydig cells had disappeared from the interstitium. Within the first 6 h after EDS injection, the levels of the proapoptotic proteins Fas and Fas-ligand increased significantly and remained elevated up to 18 h after EDS administration (ref. *53*; Fig. 4).

Activation of Fas led to the proteolytic cleavage of pro-caspase-3 into active caspase-3, one of the executioner caspases that plays an essential role in the process of apoptosis. By 12 h post-EDS, pro-caspase-3 protein levels had decreased significantly, whereas the two cleaved active caspase-3 isoforms now became detectable and their levels increased up to 24 h after EDS administration *(54)*. The latter data expands the initial morphological observations that EDS induced Leydig cell death by activating an apoptotic pathway.

The initially observed cytotoxic effects of EDS on Leydig cell morphology and function, led to an increased interest in the mechanism of action of EDS. The first in vitro studies demonstrated that one of the measurable effects of EDS was an inhibition of steroid production, either at a site before or after pregnenolone biosynthesis *(47,84,90)*. The exact details of the relation between the decrease in steroid production and Leydig cell death were not clear at that point of time. Subsequently, in the next few years biochemical studies showed that when Leydig cells were exposed to EDS either in vivo or in vitro, the biosynthesis of testosterone appeared to be rapidly compromised between the cyclic adenosine monophosphate activation of protein kinase A and the cholesterol side chain cleavage ($P450_{scc}$) enzyme *(105)*. This enzyme, localized inside the mitochondria, cleaves cholesterol to form a substrate for steroid synthesis. In a more recent study, it was reported that EDS impaired the mitochondrial electrochemical potential (δ-psi), thus, affecting the import of steroidogenesis acute regulatory protein (StAR) into the mitochondria *(4)*.

StAR regulates the transport of cholesterol across the mitochondrial membrane; in the absence of StAR

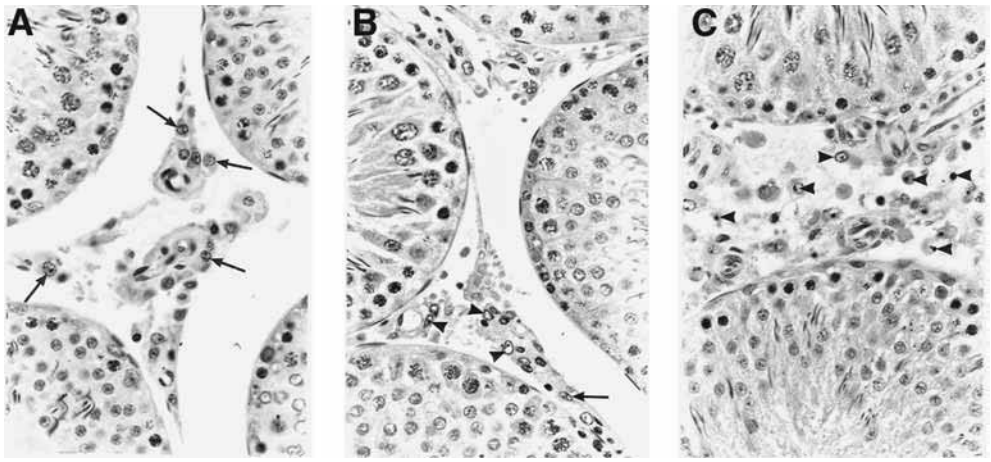


Fig. 3. Testicular histology of a control rat and animals 8 and 16 h after EDS administration. Tissues were fixed in Bouins, embedded in JB4 resin and stained by the PAS reaction and Gill's hematoxylin. **(A)** Testis of a control rat, healthy Leydig cells are indicated by arrows. **(B)** Section of a testis of an animal 8 h post-EDS injection. Leydig cell nuclei that show condensation of the nuclear chromatin, a morphological sign of apoptosis, are indicated by arrowheads. **(C)** Testis of an animal 16 h after a single injection of EDS. Note that most Leydig cells shown by now morphological signs of apoptosis.

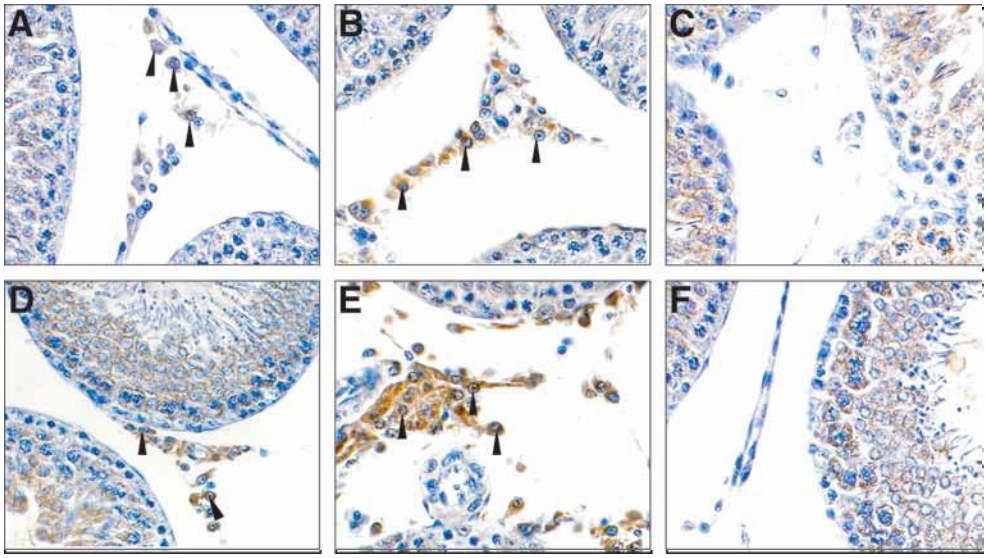


Fig. 4. Immunolocalization of Fas **(A–C)** and Fas ligand **(D–F)** in paraffin-embedded testicular tissue of adult control rats **(A,D)** and rats sacrificed at 6 h **(B,E)** and 48 h **(C,F)** post-EDS. The Fas and Fas-ligand antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). In the control testis Fas staining **(A)** in Leydig cells (brown staining of cytoplasm, indicated by arrowheads) was moderate, whereas Fas ligand stainning in Leydig cells (**D**, brown staining of cytoplasm, indicated by arrowheads) was slightly more intense. Within 6 h following EDS administration Fas **(B)** and Fas-ligand **(E)** immunostaining in Leydig cells increased concomitantly with condensation of the nuclear chromatin in these cells 48 h after EDS administration Fas **(C)** and Fias ligand **(E)** staining had disappeared from the interstitum. All sections were counterstained with Mayer's hematoxylin. (Please *see* color version of this figure in color insert following p. 180.)

steroidogenic activity is severely reduced *(105–107)*. Concomitantly with the EDS-induced decrease in testosterone biosynthesis, an increase in intracellular glutathione levels was observed. When this increase was inhibited by buthion sulfoximine administration, the cytotoxic effects of EDS on rat Leydig cells were abolished *(108,109)*. Taken together, these data suggested that the mechanism by which EDS exerted its cytotocic effects on Leydig cells seemed to involve impairment of δ-psi and StAR synthesis or functioning,

leading to a loss in steroidogenic capacity *(4)* as well as a change in intracellular glutathione levels. Whether these increased glutathione levels affect δ-psi and StAR protein levels or function is at present not clear.

REGENERATION OF THE ADULT-TYPE LEYDIG CELL POPULATION AFTER EDS ADMINISTRATION

Proliferation and Differentiation of Leydig Cell Precursors After EDS Administration

In this section, different aspects of Leydig cell regeneration following EDS administration will be discussed. Within the first few days after EDS administration, concomitant with the massive apoptosis of the mature Leydig cells, a wave of proliferation of mesenchymal-like interstitial cells was observed *(68,71,74,76)*. Pulse-chase experiments, using ^{3}H-thymidine incorporation during the S-phase of the cell cycle as a marker for cell proliferation, made it possible to follow the developmental pathway of these proliferating mesenchymal-like cells. Some of the cells that incorporated the label during the first 4 d following EDS administration, developed into new Leydig cells between days 7 and 14 after EDS *(71)*. Similar experiments carried out in (pre)pubertal rats demonstrated that the initiation of the formation of the adult-type Leydig cell population also started with the proliferation of mesenchymal-like interstitial cells, which then developed in Leydig cell progenitors *(20)*.

As a result of the destruction of the Leydig cell population after EDS administration, the negative feedback control of testosterone on pituitary LH release no longer occurs. As a consequence, high plasma levels of LH accompany the first 14 d of the regeneration process *(58,64,91)*. Because the LH analog hCG has been shown to stimulate both proliferation, and differentiation of LH receptor expressing Leydig cell precursors in the (pre)pubertal and adult testis *(68,72,110,111)*, the possibility was investigated whether the elevated LH levels in the EDS-treated animals were responsible for the increased proliferation of interstitial cells. To prevent the rise in LH levels after EDS, adult rats received a testosterone implant before EDS treatment. In these animals, the pattern of proliferation of interstitial cells was identical to rats that had received EDS only, suggesting that elevated levels of LH were not essential for the stimulation of interstitial cell proliferation following EDS administration *(68,69)*. Even in the absence of LH in hypophysectomized rats, cell proliferation was stimulated *(70)*. The latter study further demonstrated that the first part of the differentiation process of the precursor cells along the Leydig cell lineage could take place in the absence of LH and other pituitary hormones *(70)*. Interestingly, hypophysectomy followed by EDS treatment did not interfere with the regeneration of the Leydig cell population in a lower vertebrate, the frog *Rana esculenta* *(101)*. These studies suggest a role for locally produced growth factors in the regulation of these developmental processes in both mammals and lower vertebrates.

Further support for a role of locally produced factors in the process of Leydig cell regeneration came from experiments, in which unilaterally cryptorchid rats were treated with EDS. Although, both testes were exposed to the same amount of LH, new Leydig cells developed more rapidly in the intra-abdominal testis than in the scrotal testis *(60,66)*. When hypophysectomized rats, in which the seminiferous epithelium was severely affected, were treated with EDS in combination with hCG, the first regenerating Leydig cells were also observed close to the seminiferous tubules *(70)*. It cannot be excluded that the higher temperature in the abdominal testis compared with the scrotal testis affected the rate of the regeneration process. Several other studies have reported that in case the seminiferous epithelium was disrupted because of testosterone ablation, a significantly higher number of regenerating Leydig cells was observed close to the tubules, compared with animals in which the effect on the seminiferous tubules was less clear *(62,72,112)*. These data suggest that locally produced factors, released as a result of damage to the seminiferous epithelium, enhanced the differentiation of precursor cells into new Leydig cells.

Although, the factors that stimulate precursor cell proliferation and differentiation are largely unknown, FSH and estradiol were reported to have either no effect or an inhibitory effect on these processes. In contrast to what has been observed in the (pre)pubertal hypophysectomized rat, in which FSH treatment stimulated the formation of adult-type Leydig cells *(34–36)*, FSH treatment of adult hypophysectomized EDS-treated rats did not result in the formation of new Leydig cells, not even after 5 wk of treatment *(64)*. A more recent study confirms these observations using a more elegant approach. In this study, the formation of steroid producing Leydig cells appeared to be blocked when EDS-treated rats received injections with LH neutralizing antibodies. This treatment led to undetectable LH levels in serum although FSH levels were not or only marginally affected *(113)*. Hence, even under these conditions FSH was not able to stimulate the formation of new Leydig cells in the absence of

LH. An explanation for these different effects of FSH on adult-type Leydig cell development in these two models could be that, in the (pre)pubertal testis FSH stimulated indirectly through factors produced by the Sertoli cells, the proliferation and/or differentiation of adult-type Leydig cells. On the other hand, in the adult rat testis, the Sertoli cells have undergone complete maturation, and might not be able to respond to FSH treatment in the same way as in the (pre)pubertal testis. This assumption implicates that minor differences might exist in the testicular microenvironment between the (pre)pubertal testis and the adult testis following EDS treatment that could affect adult-type Leydig cell development. In the following sections of this chapter, it will become apparent that although these minor differences might indeed exist, they do not have a significant influence on adult-type Leydig cell development.

When rats were injected daily with estradiol between days 5 and 30 after EDS administration, the regeneration of the Leydig cell population was delayed *(114)*. In comparison, treatment of 5-d-old rats with estradiol resulted in inhibition of the development of the adult-type Leydig cell population. Even at the age of 60 d, only atrophic Leydig cells were present in the interstitial compartment *(115)*. As both Leydig cell precursors and adult-type Leydig cells were shown to express estrogen receptor messenger ribonucleicacid (mRNA), estradiol could affect precursor cell as well as Leydig cell development directly *(116)*. Although the effects of estradiol in neonatal rats and adult EDS-treated rats show many similarities, one has to keep in mind that there exists a major difference between these two groups of animals. In the neonatal/(pre)pubertal testis the Sertoli cells are the source of estrogen production *(15)*, whereas in the adult testis the Leydig cells have taken over the role of the Sertoli cells and have become the major source of estrogen synthesis *(117)*. Furthermore, the doses of estradiol used in these experiments to induce these inhibitory effects were extremely high *(114,117)*. As lower doses of estradiol were not used, it is not clear whether the inhibitory effect of estradiol on Leydig cell regeneration after EDS is of any physiological significance.

Several studies have indicated that testicular macrophages influence the process of Leydig cell regeneration after EDS, presumably through the secretion of growth factors and/or cytokines. During the first few days post-EDS injection, at the time when the Leydig cells undergo massive apoptosis, the intratesticular number of macrophages increased rapidly. Between days 7 and 14 post-EDS injection macrophage numbers declined again to control levels *(1,59,62,118)*. When macrophages were selectively depleted in one testis by an intratesticular injection of dichloromethylene diphosphonate-containing liposomes either before or within 10 d after EDS administration, Leydig cell regeneration was strongly inhibited. Even 30 d post-EDS injection the percentage of Leydig cells in the macrophage depleted testis was only between 1 and 3% of that in the contra lateral, vehicle-treated testis *(119,120)*. In the macrophage-depleted testis, an early rise in the concentration of small mononuclear, lymphocyte-like cells were observed, as well as a higher influx in circulating monocytes and variable inflammatory infiltrates *(121)*. These infiltrating cells could very well secrete factors that inhibit the proliferation and differentiation of Leydig cell precursors, thus, severely affecting the regeneration of the Leydig cell population after EDS. On the other hand, in the contra lateral, vehicle-treated testis *(121)*, or in rats that received only an EDS injection *(1,59)* in which the rise in infiltrating macrophages could occur undisturbed, macrophages would readily be able to phagocytose the remnants of the apoptotic Leydig cells. This could facilitate their activation leading to an increased secretion of cytokines, such as interleukin (IL)-1β *(122)*. IL-1β is a cytokine that has been shown to be a potent mitogen for Leydig cells isolated from the (pre)pubertal testis *(123)* and possibly also stimulate the proliferation of Leydig cell precursors. Hence, one could hypothesize that following EDS administration testicular macrophages become activated and start to produce large amount of IL-1β, which might then stimulate proliferation and possibly also differentiation of Leydig cell precursors. However, without furthermore research, a role for other locally produced factors cannot be excluded.

One of the factors that might play a role in the regulation of the proliferation and differentiation of Leydig cell precursors after EDS is stem cell factor (SCF)/kit ligand (KL)-1 (soluble kit). By administering an anti-*c*-kit antibody to EDS-treated rats and thus inhibiting activation of kit receptor, Yan and colleagues *(124)* showed that Leydig cell precursor cell proliferation was partially inhibited. Differentiation of these cells into new Leydig cells was not blocked by the antibody treatment. When precursor cells were isolated at different time points after EDS and treated with SCF/KL-1 their proliferation increased significantly. These results implicate a role for SCF/KL-1 in stimulation of precursor cell proliferation after EDS administration *(124)*. In a more recent study using c-kit-deficient mutant rats, the observations by Yan and coworkers were questioned. In this study, it was demonstrated that

the absence of a functional kit receptor did not influence the regeneration process after EDS, using the relative weight of the androgen-dependent accessory sex organs, seminal vesicle, and prostate as read out for Leydig cell regeneration *(125)*. These authors did not determine the proliferative activity of the Leydig cell precursors during the first few days after EDS nor investigated whether the Leydig cell population had completely regenerated up to its original size. Hence, it is still very well possible that SCF/KL-1 is involved in the regulation of Leydig cell precursor proliferation. Inhibition of *c*-kit might affect the size of the regenerating Leydig cell population, although it does not block the regeneration process, suggesting that other factors are involved as well.

One of these other candidate factors that might play a role in the regulation of precursor cell proliferation following EDS administration, is Müllerian inhibiting substance (MIS), a factor that is produced by the Sertoli cells. Intratesticular administration of MIS to EDS-treated rats resulted in a delay in precursor cell proliferation and their differentiation into new Leydig cells. By day 35 after EDS, at a time when under normal conditions the regeneration of the Leydig cell population had been nearly completed, the number of Leydig cells per testis was still one-third lower compared with the controls which received only a single injection of EDS *(126)*.

On the other hand, daily treatment with high doses of thyroid hormone (tri-iodothyronine [T_3]) advanced Leydig cell regeneration after EDS administration *(56)*. Functional thyroid hormone receptors were demonstrated to be present in interstitial cells; although, the origin of the cell type expressing this receptor, Leydig cells or their precursors, is unknown *(127)*. Hence, it is not clear whether thyroid hormone influenced Leydig cell regeneration directly or indirectly through the Sertoli cells *(128)*. In contrast to hyperthyroidism, hypothyroidism inhibited precursor cell differentiation into new Leydig cells following EDS administration. As a consequence, the number of mesenchymal-like Leydig cell precursors was slightly but significantly elevated 21 d after EDS administration. At this point of time, no new Leydig cells had developed *(56)*. Expanding the experiment to, for instance, 50–60 d after EDS administration would provide an answer to the question whether the absence of thyroid hormone merely delays Leydig cell regeneration after EDS, or completely blocks this process. The latter would implicate that thyroid hormone is essential for adult-type Leydig cell development. The effects of hyperthyroidism and thyroid hormone depletion on Leydig cell regeneration after EDS were more or less identical to what was observed when neonatal/(pre)pubertal rats were made hyper- or hypothyroid *(26,129)*.

Although, precursor cells in testes of EDS-treated hypophysectomized rats could undergo some differentiation in the absence of LH, development into adult-type Leydig cells required hCG administration for several days *(70)*. This indicates that the presence of functional LH receptors is essential during the final part of the developmental pathway that precursor cells undergo before they differentiate into 3β-HSD expressing Leydig cells. Initial studies in which Northern blot analysis of poly $(A)^+$ RNA isolated from testes of hypophysectomized rats 4 mo after EDS administration was performed, showed indeed that Leydig cell precursors contained the five different LH receptor transcripts described previously in the (pre)pubertal and adult rat testis (refs. *130–135*; Fig. 5). However, hypophysectomy followed by EDS treatment drastically changed the pattern of the LH/CG receptor mRNA content when compared with untreated intact control rats. The amounts of the 7, 4.2, 2.5, and 1.2 kb transcripts in the EDS-treated rats were considerably lower than in the intact control, but still measurable. The amount of the 1.8 kb transcript was slightly higher than in the intact control (Fig. 5). Within 4 h after administration of 100 IU of hCG to these hypophysectomized EDS-treated rats, a significant increase in the amount of the 1.8 kb transcript was observed (Fig. 5). When hCG was administered for 7 d, new steroid producing Leydig cells could be identified *(70,135)*, and concomitantly, an increase in the amounts of all transcripts except the 1.8 kb transcript was observed (refs. *135*; Fig. 5). More or less comparable results were obtained when total RNA was isolated from testes of intact EDS-treated rats, reversely transcribed and amplified by the polymerase chain reaction *(136)*.

In a more recent study, it was demonstrated that Leydig cell precursor cells do not always express detectable levels of all LH receptor transcripts. During the first few days after EDS the precursor cells mainly express the 1.8 kb LH receptor transcript. Expression of the full length transcript coding for the functional LH receptor was first measured around day 10 after EDS administration *(136)*. This study offers an explanation for the results of Veldhuizen and colleagues *(135)* in hypophysectomized rats, who in contrast to the study by Abney and Zhai *(137)* determined the presence of LH receptor mRNA transcripts 4 mo after EDS administration. This lag in time left between EDS administration and the time of euthanasia gave the precursor cells ample opportunity to acquire all LH receptor mRNA transcripts at detectable levels. In another

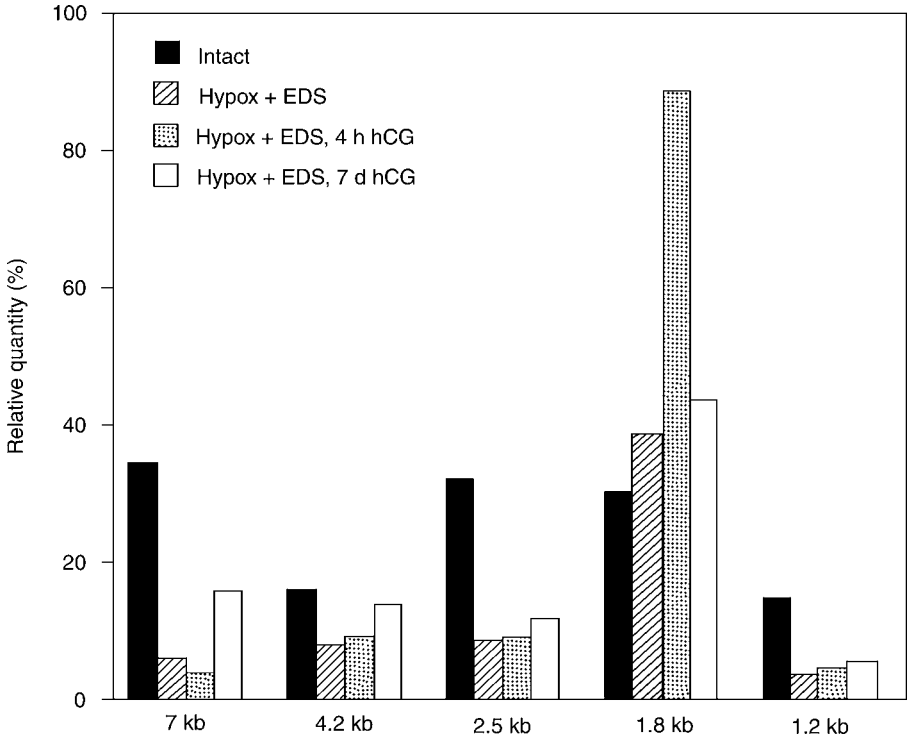


Fig. 5. Northern blot analysis of testicular tissue for the detection of LH receptor mRNA transcripts. Poly $(A)^+$ mRNA was isolated from testes of intact adult rats, hypophysectomized-EDS-treated adult rats (hypox + EDS), of hypophysectomized EDS-treated rats which received one injection of 100 IU of the LH analog hCG and sacrificed 4 h later (hypox + EDS, 4 h hCG) and of hypophysectomized EDS-treated adult rats which received daily injections of 100 IU hCG for 7 d before being sacrificed (hypox + EDS, 7 d hCG). The oligonucleotide probe for the rat LH receptor was hybridized with Northern blots of rat testiscular poly $(A)^+$ mRNA. Hybridization occurred only with RNA species of correct molecular sizes according to the literature *(130)*. The integrated optical densities of the five different transcripts were determined by IBAS image processing of the autoradiograms (IBAS image analysis system; Zeiss/Kontron, Eching, Germany). The integrated optical densities of the LH receptor mRNA species were normalized for those of D-glyceraldehyde-3-phosphate dehydrogenase and plotted as percentage of relative quantities (adapted from ref. *135*).

study it was demonstrated that the expression of the 1.8 kb LH receptor transcript appeared to be influenced by the temperature of the microenvironment. These authors showed that in cryptorchid EDS-treated animals the amount of the 1.8 kb transcript was considerably lower at day 5 after EDS compared with the scrotal testis *(138)*.

There existed some controversy about the functional significance of the different LH receptor transcripts and whether one or more transcripts would code for the full length, functional receptor. Koo and coworkers *(139)* have demonstrated that the 7 kb transcript, which could be detected in Leydig cell precursors at day 10 after EDS *(136)* as well as in the testis of hypophysectomized EDS-treated rats *(135)*, was the only LH/CG receptor mRNA transcript which did not carry any intronic polyadenylation sites. Therefore, this 7 kb transcript is the only one that can code for the full length LH receptor protein. The other transcripts represent truncated forms of the LH/CG receptor and are presumably not involved in signal transduction, as they fail to bind hormone when transfected in intact cells *(139)*.

Although, LH receptor mRNA could be detected by either Northern blot analysis or reverse transcriptase-polymerase chain reaction *(135–137)*, it was impossible to demonstrate significant ^{125}I-hCG binding in interstitial cell preparations isolated from intact rats during the first 2 wk after EDS injection *(3,66,140)*. Low levels of specific binding were detected for the first time in isolated precursor cell fractions from day 15 post-EDS onward *(141)*. A further increase in specific binding of ^{125}I-hCG was observed with the development of new Leydig cells between days 14 and 21 after EDS *(65,66,141)*. The lower sensitivity of the ^{125}I-hCG binding assay in comparison with the detection of LH/CG mRNA might offer an explanation for this discrepancy. The latter data are supported by the demonstration of LH receptor protein in Leydig cell

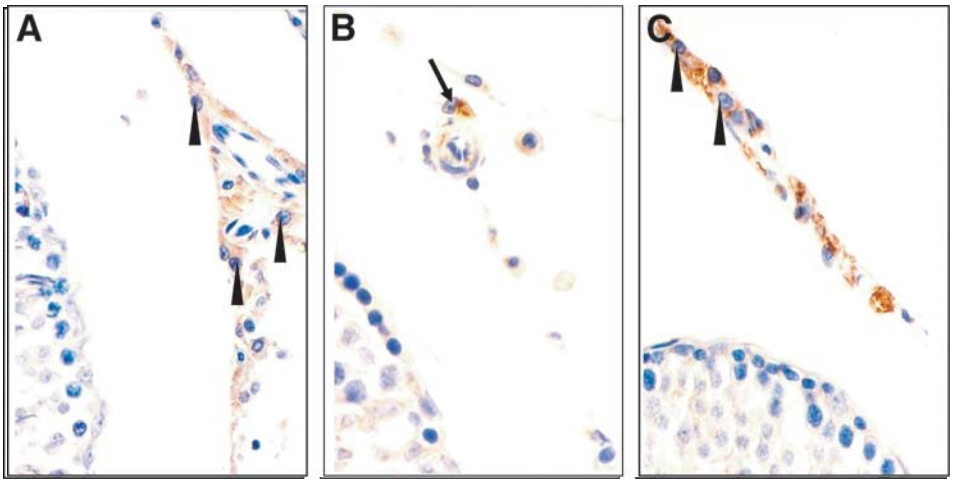


Fig. 6. Immunohistochemical localization of LH receptors in paraffin-embedded testicular tissue of adult rats before and after EDS administration. The LH receptor monoclonal antibody (P1B4) was obtained from Dr. J Wimalasema (Dept. of Obstetrics and Gynecology, University of Tennessee, Knoxville, TN). The antibody was raised against purified rat LH receptors as described by Indrapichate et al. *(142)* and has been used before to demonstrate LH receptors on Leydig cells and their precursors *(72)*. **(A)** Immunohistochemical staining of a testis section of a control rat. Positively stained Leydig cells (brown cytoplasm) are indicated by arrowheads. **(B)** Section through a testis of a rat that had received an injection of EDS 8 d before. Leydig cells are not present at this stage, but LH receptor immunoreactivity can be detected in a mesenchymal-like precursor cells (arrow). **(C)** Rat testis 51 d after EDS administration. The Leydig cell population has regenerated and many LH receptor expressing Leydig cells can be observed. All sections were counterstained with Mayer's hematoxylin. (Please *see* color version of this figure in color insert following p. 180.)

precursors as early as 3 d after EDS administration, using a monoclonal antibody raised against the rat LH receptor (ref. *72*; Fig. 6). This antibody has been raised against isolated and purified LH receptor protein *(142)* and therefore, cannot discriminate between the full length receptor protein and proteins transcribed from the truncated LH receptor transcripts.

Although, our knowledge concerning the early phases of precursor cell proliferation and differentiation in the Leydig cell lineage is still increasing, the consensus remains that many of the factors involved in this developmental process are still unknown. LH/hCG can stimulate this process in the presence of testicular macrophages *(64,68,119,120)*, but part of this process can also take place in the absence of LH and other pituitary hormones, presumably under the influence of systemic and locally produced factors. In order for the precursor cells to develop into adult-type Leydig cells, the former cells need to acquire LH receptors and become exposed to LH in the presence of testicular macrophages. The future will reveal whether thyroid hormone is also necessary for this process to proceed.

Proliferation and Differentiation of the Newly Formed Leydig Cells (Leydig Cell Progenitors) After EDS Administration

In the intact adult rat, the first morphologically recognizable and 3β-HSD-positive Leydig cells developed between days 7 and 14 after EDS administration *(1–3,58,59,62–64,71,73,74,76)*. At the same time, these newly formed Leydig cells started to proliferate actively. Peak values of labeled Leydig cells that have incorporated ^{3}H-thymidine during the S-phase of the cell cycle, as well as mitotic figures representing cells in the M-phase of the cell cycle, were found at days 21 and 22 post-EDS injection, respectively *(71,74,76)*.

The factors involved in the control of the proliferative activity of regenerating Leydig cells are largely unknown. Increased plasma LH/hCG levels have been reported to enhance Leydig cell proliferation in both the (pre)pubertal and the adult rat *(27,36,68,110,111)*. A question that arose from these studies was whether elevated LH levels were also required for the stimulation of Leydig cell proliferation during the regeneration process. In the (pre)pubertal testis the peak values in Leydig cell progenitor proliferation occurred in the absence of elevated LH levels *(20,143,144)*, suggesting that although LH is essential for the final steps of Leydig cell precursor differentiation in these young animals, it is not important for Leydig cell proliferation. Plasma LH levels were elevated up to 14 d after EDS because of the absence of testosterone-producing Leydig cells. Between days 14 and 21 post-EDS injection, plasma LH levels started to decrease again as a result of the formation of new testosterone producing

Leydig cells. By day 21 after EDS administration, plasma LH levels had returned to the pretreatment range *(58,59,64)*. Because LH levels were no longer elevated at the time when the newly formed Leydig cells initiated their proliferation, it seems likely that other factors trigger this wave of proliferation.

The described similarities between the development of the adult-type Leydig cell population in the (pre)pubertal testis and the regeneration of the Leydig cell population following EDS administration, have prompted investigators recently to use the same nomenclature to describe Leydig cell development in the (pre)pubertal testis and adult testis after EDS administration. Briefly, precursor cells are described as mesenchymal-like Leydig cell precursors that express LH receptor mRNA and protein, and which develop into Leydig cell progenitors which still have an elongated shape but now also express steroidogenic enzymes such as 3β-HSD. These cells subsequently differentiate into immature Leydig cells, which have several morphological characteristics in common with mature adult-type Leydig cells. Immature Leydig cells are somewhat smaller than mature adult-type Leydig cells, contain lipid droplets in their cytoplasm and have initially 5α-reduced androgens as major secretory product. These cells finally, differentiate in large mature adult-type Leydig cells that have as major secretory product testosterone (for more information *see* reviews by Mendis-Handagama *[21]* and Haider *[22]*).

So far not much information has become available concerning the factors that might stimulate the proliferation of the progenitor Leydig cells after EDS administration. The majority of the studies investigating the role of growth factors in this process have been carried out in (pre)pubertal animals. In the next section of this chapter, where possible, a brief comparison will be made between adult-type Leydig cell development in the (pre)pubertal rat and the adult rat following EDS administration (other chapters in this book will review the development of adult-type Leydig cells in the [pre]pubertal testis more extensively).

Role of Growth Factors

Several growth factors have been demonstrated to stimulate DNA synthesis in progenitor Leydig cells in vitro, such as TGF-α, IGF-1, and IL-1β *(123,145,146)*.

IGF-1 AND TGF-α

When progenitor cells were isolated from (pre)pubertal rat testes and incubated concomitantly with a low dose of LH and a mixture of TGF-α and IGF-1, a synergistic effect on the stimulation of DNA synthesis became apparent *(145,146)*. IGF-1 alone could also stimulate progenitor DNA synthesis, although, these effects were less pronounced *(145,147)*. The importance of IGF-1 in Leydig cell development was furthermore established recently in a study using IGF-1 knockout mice. In the absence of IGF-1 the adult-type Leydig cell population was reduced in size. When exogenous IGF-1 was administered to these mutant mice, Leydig cell precursor cell, progenitor cell, and immature Leydig cell proliferation were enhanced *(148)*. In order to understand the physiological significance of this in vitro data, a study was performed to address whether these peptides were present in the interstitial compartment or the seminiferous tubules. Using immunohistochemical techniques, it was demonstrated that TGF-α was indeed present in the interstitium of the (pre)pubertal rat testis. At 21 d of age approx 50% of the progenitor Leydig cells stained positively for this growth factor *(149)*. As the wave of proliferation proceeded, all progenitor Leydig cells acquired intense staining for TGF-α *(20,149)*. At present it is not known whether these progenitor cells themselves produce TGF-α, or whether they accumulate this growth factor by binding it from the interstitial fluid. IGF-1 is also present in progenitor Leydig cells during the (pre)pubertal period *(150)* and Leydig cells have receptors for this growth factor *(151)*.

Growth factors like TGF-α and IGF-1 are locally produced not only in the (pre)pubertal testis but in the late-pubertal and adult testis as well. TGF-α mRNA was expressed by Sertoli cells, peritubular/myoid cells, and Leydig cell progenitors *(150,151)*. Sertoli cells and peritubular/myoid cells also secrete IGF-1 *(152–155)*. As Leydig cells possess both epidermal growth factor receptors to which TGF-α can bind *(156)*, and IGF-1 receptors *(153,157)*, the TGF-α and IGF-1 produced by the Sertoli cells and peritubular/myoid cells could play a role in the regulation of Leydig cell progenitor proliferation during the regeneration period following EDS administration.

The possible role of TGF-a in the regulation of Leydig cell regeneration after EDS was furthermore studied by determining the cellular localization of this growth factor at different time points after EDS administration. No TGF-a immunoreactivity could be detected in the interstitium 8 d post-EDS administration when all Leydig cells had disappeared (ref. 72; Fig. 7). However, when the Leydig cell population started to regenerate between days 15 and 22 after EDS administration, both TGF-a positive and negative Leydig cell progenitors were observed (Fig. 7). Between days 29 and 35 post-EDS injection, all Leydig cells acquired

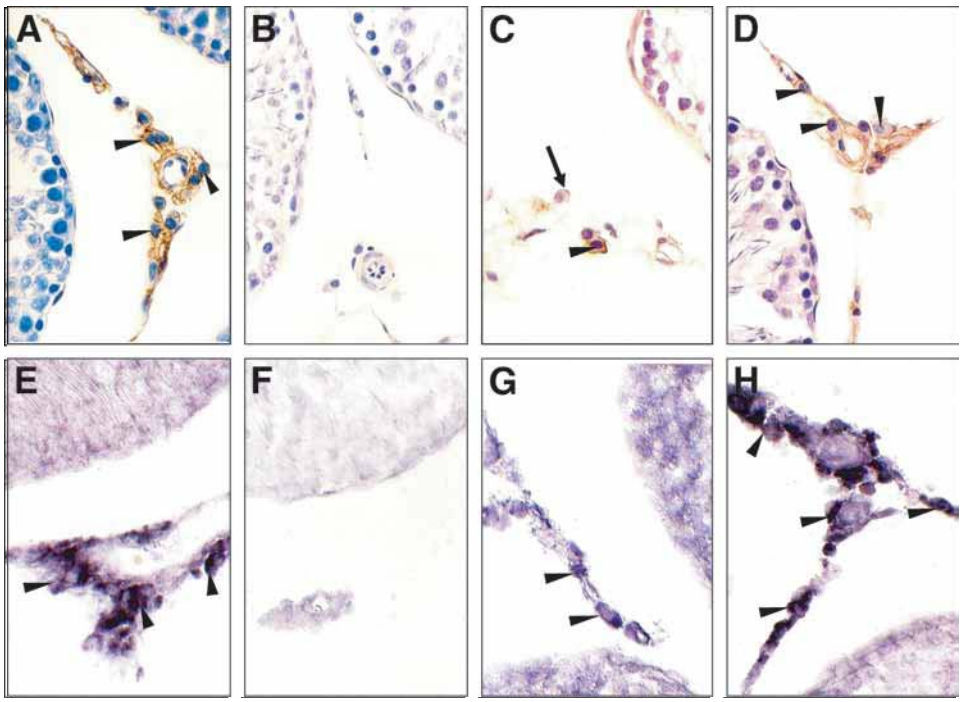


Fig. 7. Immunohistochemical localization of TGF-α and *in situ* hybridization for the presence of relaxin-like factor (RLF) mRNA in paraffin-embedded testicular tissues of adult rats following EDS administration. The TGF-α monoclonal antibody was a gift from Dr. JE Kudlow (University of Alabama, Birmingham, AL). The antibody was raised against a synthetic peptide that consisted of the carboxyl terminal 17 amino acids of rat TGF-α. The TGF-α antibody, which does not show cross reactivity with epidermal growth factor, has been used previously for the localization of TGF-α in the rat pituitary, ovary, and testis *(72,200,201)*. The rat Leydig cell-specific RLF cDNA was cloned from a rat testis cDNA library, as has been described by Spiess et al. *(202)*. **(A,E)** Sections though a control testis, TGF-α-positive Leydig cells (**A**, brown stained cytoplasm) and RLF mRNA expressing Leydig cells (**E**, blue colored cytoplasm) are indicated by arrowheads. **(B,F)** Eight days after EDS administration the testis is still depleted of Leydig cells, no TGF-α positive cells or RLF mRNA expressing cells can be detected. **(C,G)** Fifteen days post-EDS injection the first new progenitor Leydig cells can be discerned. Some of these Leydig cell progenitors stain positively with the TGF-α antibody (**C**, arrowhead), whereas other progenitor cells do not contain TGF-α protein yet (arrow). All progenitor cells express RLF mRNA **(G)**. **(D,H)** Fifty one days after EDS administration the Leydig cell population has more or less completely regenerated and many TGF-α positive adult-type Leydig cells **(D)** are present, as well as RLF mRNA expressing adult-type Leydig cells **(H)**. The testicular sections shown in **A–D** have been counterstained with Mayer's hematoxylin. (Please *see* color version of this figure in color insert following p. 180.)

this growth factor, implying that during their phase of growth and differentiation, progenitors Leydig cells could be influenced by TGF-a (ref. 72; Fig 7), a situation comparable with the pubertal rat testis (71,145,146).

The hypothesis that Sertoli cells, peritubular/myoid cells, and possibly the regenerating Leydig cell progenitors themselves secrete factors that stimulate adult-type Leydig cell proliferation, was supported by the following observations. Interstitial fluid collected from rat testes between days 21 and 28 after EDS administration, when Leydig cell progenitor proliferation was maximally stimulated, appeared to be highly mitogenic to Balb/c 3T3 cells in vitro *(71,74,76,158)*. Although, the exact nature of these growth promoting factors in interstitial fluid was not determined, the presence of the growth factors TGF-α and IGF-1 during progenitor proliferation and differentiation in the (pre)pubertal testis, and in the adult testis during the regeneration process after EDS administration, determines that this process is controlled at least in part by these factors.

Interleukin-1β

In vitro studies demonstrated that progenitor Leydig cell DNA synthesis can be further enhanced by the addition of IL-1β *(123)*. Although, IL-1 receptors have been localized in the interstitial compartment *(159)* and Leydig cells in the adult testis have been shown to express the IL-1β gene *(160)*, the role of this cytokine in testicular development has been questioned. In IL-1 type I receptor knockout mice Leydig cell development and function, as well as spermatogenesis were not affected by the absence of IL-1 receptor signaling *(161)*, suggesting that, although IL-1β has a significant

stimulatory effect on progenitor proliferation, its role can be easily taken over by other factors. Whether macrophage-derived IL-1β is also involved in the process of Leydig cell regeneration after EDS administration has not been determined yet.

Müllerian Inhibiting Substance

Progenitor and immature Leydig cells furthermore express the MIS type II receptor during the (pre)pubertal period *(162)*. Mutation of MIS resulting in complete abolishment of MIS production caused Leydig cell hyperplasia in mice at 2 mo of age *(163)*, implicating that MIS which is normally produced by the Sertoli cells, might act as a local regulator of adult-type Leydig cell development. On the other hand, overexpression of MIS during the (pre)pubertal period resulted in a reduction in Leydig cell numbers during adulthood as compared with wild-type animals *(164)*. Similar results were obtained during Leydig cell regeneration following EDS treatment of adult rats *(127)*, suggesting that MIS might inhibit the proliferation of progenitor cells.

Thyroid Hormone

Besides MIS, T_3 also affected progenitor cell proliferation. Both in the (pre)pubertal rat testis and in the adult testis during the regeneration process following EDS treatment progenitor proliferation was stimulated significantly by daily injections with T_3 *(26,56)*.

Transforming Growth Factor-β

Whereas, LH and the growth factors TGF-α, IGF-1, and IL-1β stimulate DNA synthesis in Leydig cells from the (pre)pubertal testis, TGF-β was the only factor tested so far that could attenuate the proliferative response of Leydig cell progenitors to these stimulatory factors *(146,165)*, presumably through a direct effect of the latter growth factor through activation of its receptors *(166,167)*. Immunohistochemical localization studies for the presence of the different types of TGF-β in rat testis showed that at 7 d of age all (fetal) Leydig cells contained TGF-β_1. With the differentiation of Leydig cell progenitors, the number of positively stained cells declined and by 21 d of age approx 50% of the progenitors were positive for this growth factor *(146,168)*. As the wave of proliferation and differentiation of progenitor Leydig cells further progressed, and these cells differentiated into immature Leydig cells, the immunostaining for TGF-β_1 further decreased. By the age of 35 d no TGF-β_1 immunoreactivity could be detected in the interstitial space anymore, whereas TGF-β_2 protein levels were around the detection limit of the immunohistochemical assay *(168)*. Based on this knowledge it is postulated that LH stimulates Leydig cell proliferation in the (pre)pubertal testis only in the presence of growth factors like IGF-1, TGF-α, and IL-1β. The latter factor is either produced by the Leydig cells themselves or released by the testicular macrophages and taken up by the Leydig cells. The stimulatory effects of these factors on cell division are kept in abeyance by TGF-β *(27)*. Whether TGF-β plays a regulatory role in the regeneration process after EDS, as it does in the (pre)pubertal testis, is at present not known.

Mast Cells

Several groups have observed the appearance of mast cells in the interstitial compartment during Leydig cell regeneration after EDS *(59,76,169)*. Gaytan and colleagues *(76,169)* have reported that at the time when the mitotic activity of the newly developed Leydig cell progenitors was high, the proliferative activity of these infiltrating mast cells was also elevated. These authors suggested that presumably common regulatory factors were involved in the enhancement of proliferation in both cell types *(76)*. Although this might indeed be the case, the presence of infiltrating mast cells is not a general phenomenon in the regenerating testis after EDS administration. In studies using intact Wistar rats, Molenaar, Rommerts, and Teerds *(2,64,71)* never observed the appearance of mast cells or inflammatory cells concomitantly with the regeneration of Leydig cells following EDS administration. Therefore, the appearance of mast cells in the interstitial compartment might be related to the degree of inflammation as a result of EDS treatment *(72)*, a phenomenon that was regularly observed in Sprague Dawley rats (e.g., ref. *61*), but rare in most strains of Wistar rats. Studies investigating the effects of EDS on Leydig cell regeneration have in general been carried out in Wistar rats. Taken together, these data suggest that the presence of mast cells does not seem to play an essential role in the process of Leydig cell regeneration following EDS administration.

Steroic Production

Progenitor Leydig cells were furthermore characterized by a specific pattern of steroid production. The major steroids secreted during progenitor differentiation were 5α-reductase steroids. As a consequence 5α-reductase activity increased significantly to more than pretreatment levels, reaching a maximum value between days 21 and 35 after EDS *(92,170,171)*. This situation is comparable with the (pre)pubertal testis where the primary androgens produced between days 20 and 35 after birth are androstenedione and the 5α-reduced androgens, dihydrotestosterone, and 5α-androstanediol *(172)*.

Proliferation and Differentiation of the Regenerating Immature Leydig Cells

The proliferative activity of the regenerating Leydig cell progenitors following EDS administration decreased rapidly around day 35 post-EDS when these cells had differentiated into immature Leydig cells. By day 49 after EDS treatment, the proliferative activity of the immature Leydig cells had ceased *(71,74,76)*. Concomitantly the differentiation of the immature Leydig cells was initiated, as became apparent by changes in the activities of several steroid metabolizing enzymes. Both in the adult testis after EDS treatment and in the pubertal testis, 5α-reductase levels decreased rapidly after day 35 *(92,170–172)*. Around the same period, together with the rise in the number of differentiating immature Leydig cells, the testicular activity of both $P450_{scc}$ and 17α-hydroxylase ($P450_{17\alpha}$)—enzymes that play an important role in testosterone synthesis—began gradually to increase *(171,173)*. Together with the decrease in 5α-reductase activity, testosterone has now become the major androgen produced *(170,171, 173–176)*. In the pubertal rat, at the same time when 5α-reductase activity rapidly declined, 11β-hydroxysteroid dehydrogenase (11β-HSD) type I activity began to increase, whereas 11β-HSD type 2 activity, although at low levels, also became detectable *(177–180)*. Although, 11β-HSD can be used as a marker enzyme for immature Leydig cell development, its primary role is to control the toxicological effects of glucocorticoids on Leydig cells *(178–180)*. To date it is not known whether the latter enzyme follows the same developmental pattern in differentiating immature Leydig cells during the regeneration process after EDS.

Morphologically the regenerating Leydig cells after EDS administration are, as implicated earlier, very similar to the immature adult-type Leydig cells in the pubertal testis *(74,92)*. During their further differentiation, the regenerating Leydig cells obtain the morphological and functional characteristics of mature adult-type Leydig cells and, like the differentiating Leydig cells in the pubertal testis, they become sensitive to a second EDS challenge between days 70 and 140 after the first EDS injection *(63)*.

Dynamics of the Mature Adult-Type Leydig Cell Population in the Adult Testis

In the normal rat testis, the process of growth and differentiation of the adult Leydig cell population is completed around day 70 postpartum *(181)*. Although, there are a few studies in monkeys *(182,183)* and human *(184)* that reported the presence of Leydig cells which had incorporated ^{3}H-thymidine during the S-phase of their cell cycle, or rare mitotic figures corresponding to Leydig cells, it was generally assumed that the Leydig cell population in the adult testis was a stable population of cells which did not undergo turnover under normal conditions. This dogma was questioned in a pulse-chase experiment by Teerds and colleagues *(185)*, in which it was shown that the interstitial tissue of the adult rat testis still has a limited capacity to undergo cell renewal. Taking into account the 95% confidence interval the turnover time of Leydig cells in the adult testis was determined to range from 142 d to the maximum life span of the animal, whereas the turnover time of the peritubular/myoid cells (including mesenchymal-like Leydig cell precursors) was estimated to range from 85 to 257 d *(185)*.

The low turnover rate in the adult testis suggests that in the adult testis beside terminally differentiated mature Leydig cells, also a limited number of immature Leydig cells continue to be present which under certain conditions can be stimulated to proliferate. The proliferation of these immature cells is possibly inhibited by a growth factor yet to be identified. The mature terminally differentiated Leydig cells themselves might be the cells responsible for the secretion of proliferation inhibitory factors, because in the absence of Leydig cells interstitial cell proliferation is stimulated *(71,74,76)*. Factors that are produced by Leydig cells and which have been suggested to act as growth inhibitors are the steroids testosterone and estradiol. Testosterone was thought to be a growth inhibitor because of the fact that interstitial cells start to proliferate as soon as testosterone levels have become undetectable after EDS administration *(58,68,74,76)*. Estradiol has also been implicated to act as a cell growth inhibitor, as both in vivo and in vitro studies have demonstrated that treatment with a supraphysiological amount of estradiol inhibited ^{3}H-thymidine incorporation in Leydig cells of control and hCG-treated rats *(186–188)*. The physiological relevance of these studies is presently not clear and nonspecific effects of the use of extremely high doses of estradiol cannot be excluded.

The earlier data implicates the presence of growth stimulatory as well as inhibitory factors in the adult testis, resulting in a very low rate of Leydig cell renewal. As there are no indications that the size of the Leydig cell population increases with the age of the rat *(189)*, one could hypothesize that it is likely that some Leydig cell death must also occur in order to keep the size of the population in abeyance. To date, the incidence of Leydig cell death has not been determined in the normal adult testis.

Although, under normal conditions the turnover rate of interstitial cells in the adult testis is limited, it remains possible to stimulate interstitial cell proliferation independent of the elimination of the existing Leydig cell population by EDS. Daily injections with high doses of hCG (100 IU/rat/d) stimulated the proliferation of mesenchymal-like Leydig cell precursors in the normal adult testis *(68,98,111)*. In addition to enhancing precursor cell proliferation, hCG also stimulated the differentiation of these cells into Leydig cell progenitors *(68,111)*. Careful examination of the effects of daily hCG treatment showed that actually two hCG-dependent waves of precursor cell differentiation could be recognized. The first wave of differentiation was rapid (within 2 d after the start of treatment) and took place independent of cell proliferation, whereas the second wave was initiated approx 8 d following the start of treatment *(27,67)*.

The observation that hCG treatment could induce two independent waves of precursor cell differentiation implied that in the adult rat testis both stem Leydig cells, and Leydig cell precursors were present. The differentiation of LH receptor expressing precursor cells into 3β-HSD positive Leydig cell progenitors occurred rapidly, whereas the stem Leydig cells required a longer period of stimulation before they acquired precursor cell and subsequently progenitor properties. Yet, another explanation for these observations could be that the rapidly differentiating precursor cells were in fact inactive adult-type Leydig cells, which were not identified as such; a sudden rise in plasma LH/hCG levels could then induce the rapid activation of these cells into recognizable mature adult-type Leydig cells. The first hypothesis is favored, as in the case where EDS treatment was initiated 2–4 d after the start of daily hCG injections, the newly formed Leydig cells survived the EDS treatment. This suggested that these cells were in fact progenitor cells which were insensitive to EDS, and not inactive adult-type cells that under the influence of hCG had undergone rapid activation into mature adult-type Leydig cells, a cell type that is sensitive to EDS *(63,89,98)*.

Daily hCG administration not only affected precursor cell proliferation and differentiation, but also stimulated the mitotic activity of adult-type Leydig cells in both rats and monkeys *(68,111,186,190)*. This assumption was furthermore supported by the observation that when ^{3}H-thymidine was administered to rats on days 2, 3, and 4 of hCG treatment, the radioactive label appeared to be incorporated by both newly formed Leydig cell progenitors and existing adult-type Leydig cells (ref. *98*; Teerds, de Rooij, Rommerts, and Wensing, unpublished observations).

The stimulatory effects of hCG on Leydig cell proliferation were surprising when one takes into account the high doses of hCG used, cause desensitization of Leydig cells and their precursors in terms of a rapid loss of available LH/CG receptors, a decrease in LH receptor mRNA levels, and arrest of adenylate cyclase activity (*131,132,191–194*; Teerds et al., manuscript submitted). Apparently, these functional parameters are not essential for this growth process as they do not seem to influence the rise in mitotic activity of the Leydig cells.

In conclusion, although adult-type Leydig cell proliferation could only be stimulated in the presence of high levels of hCG, these results do implicate that in the adult testis not all Leydig cells have become terminally differentiated mature adult-type Leydig cells. Although, difficult to identify by morphological techniques, it is hypothesized that presumably not all immature adult-type Leydig cells differentiate into mature cells which are no longer able to proliferate anymore. Because of the presumably negligible levels of Leydig cell death in the adult testis under normal conditions, it seems to be essential to keep immature Leydig cell proliferation at extremely low levels, in order to establish a stable population of cells. Locally produced factors such as MIS *(127)*, TGF-β *(145)*, and perhaps also estradiol, are thought to play an essential role to keep the proliferative activity of these cells under normal conditions in abeyance.

AN INTEGRATED MODEL FOR LEYDIG CELL DEVELOPMENT IN THE (PRE)PUBERTAL TESTIS AND THE ADULT TESTIS AFTER EDS

The regeneration of the Leydig cell population following EDS administration shows, as indicated earlier, many similarities with the development of the adult-type Leydig cell population in the (pre)pubertal testis. The latter has been briefly summarized in this chapter and is more extensively discussed in Chapter 4. In the next paragraph an attempt will be made to integrate the knowledge concerning Leydig cell development in the adult testis and the formation of the adult-type Leydig cell population in the (pre)pubertal testis into one model for adult-type Leydig cell development (Fig. 8).

Precursor Cell Proliferation and Differentiation

LH receptor expressing Leydig cell precursors in both (pre)pubertal and adult rats originate from a stem cell population *(24)*. Proliferation of these stem cells can take place in the absence of LH, whereas precursor cell proliferation can be stimulated by growth factors as well as LH.

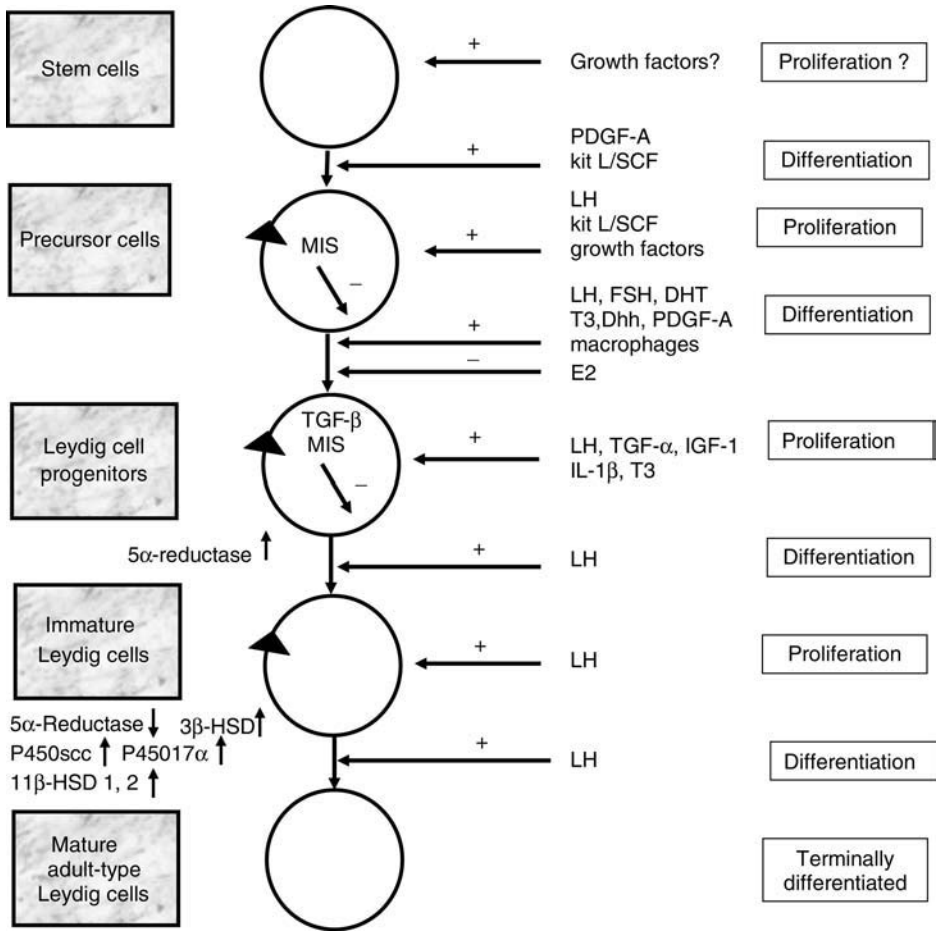


Fig. 8. Integrated model for the development of adult-type Leydig cells in the (pre)pubertal testis and in the adult testis following EDS administration. The model is discussed in "An Integrated Model for Leydig Cell Development in the (Pre)Pubertal Testis and the Adult Testis After EDS".

Gonadotropins

LH plays an essential role in the differentiation of the LH receptor expressing mesenchymal-like precursor cells. The final stage of precursor cell differentiation, when the conversion into progenitor Leydig cells takes place, is highly dependent on LH both in the (pre)pubertal and in the adult rat following EDS administration. Despite this important role of LH, there are in the (pre)pubertal testis indications that a small proportion of precursors can differentiate into progenitors Leydig cells independent of the presence of LH (ref. *195*; Teerds et al., manuscript submitted). In contrast, studies in hypophysectomized animals have demonstrated that in the regenerating testis following EDS administration differentiation of precursors into Leydig cell progenitors can only occur in the presence of LH (ref. *64*; Fig. 8).

In the pubertal rats FSH has been shown to stimulate precursor cell differentiation independent of the presence of LH, presumably indirectly by acting on the Sertoli cells (e.g., ref. *35*). In the adult EDS-treated rat, in the absence of LH, FSH was unable to stimulate this developmental process. Nevertheless, a role for FSH cannot be excluded completely, whereas depletion of FSH in the presence of elevated LH levels seemed to affect precursor cell differentiation leading to a slight arrest in the regeneration process (ref. *113*; Fig. 8).

Growth Factors

KL/SCF *(124)* has been shown to stimulate the proliferation of precursor cells in (pre)pubertal rats. The effects of KL/SCF in adult rats following EDS treatment are less clear. On the other hand, MIS inhibits precursor cell proliferation when administered following EDS administration *(126)*.

In MIS knockout mice, in the absence of MIS, Leydig cell hyperplasia was observed; suggesting that under these conditions precursor cell proliferation might be enhanced *(161)*.

The presence of interstitial macrophages appears to be essential for the conversion of precursor cells into Leydig cell progenitors, and is possibly also important for precursor cell proliferation. Both in the (pre)pubertal testis and adult testis following EDS administration, differentiation of precursor cells into Leydig cell progenitors is arrested in the absence of interstitial macrophages *(119,120)*. Because one of the genes expressed and proteins secreted by macrophages is IL-1β, it has been postulated that this cytokine together with LH might be of major importance in the process of precursor cell differentiation into progenitors (Fig. 8).

Knockout studies have demonstrated that differentiation of Leydig cell precursors into progenitor cells is dependent on the presence of two other genes, namely Desert hedgehog (Dhh) and PDGF-A. Despite normal plasma LH levels, formation of Leydig cell progenitors did not occur in the absence of Dhh or PDGF-A gene expression *(196,197)*. Because of the complexity of these studies, the role of these two factors remains to be investigated in the process of regeneration after EDS (Fig. 8).

Thyroid Hormone

Daily treatment with the thyroid hormone T_3 has been shown to advance the differentiation of precursor cells into Leydig cell progenitors in both (pre)pubertal rats and EDS-treated adult animals. In the absence of T_3, differentiation of precursor cells is either inhibited or delayed (*26,56,130*; Fig. 8).

Steroids

Besides growth factors, there are many other factors involved in the regulation of the conversion of LH receptor expressing precursor cells into 3β-HSD positive Leydig cell progenitors. The androgen dihydrotestosterone when administered in combination with LH has been shown to stimulate the conversion of precursor cells into 3β-HSD positive progenitor cells. So far, it has not been possible to repeat these experiments with precursor cells isolated from testes of EDS-treated rats (Teerds and de Boer-Brouwer, unpublished observations). It is, therefore, not clear whether this steroid plays a physiological role in this differentiation process in the (pre)pubertal rat and adult rat after EDS administration (Fig. 8).

Another steroid, which has been implicated in this part of the developmental process is estradiol *(183, 188)*. Both in the (pre)pubertal testis and in the adult testis after EDS administration, estradiol treatment caused a delay, or even inhibited the differentiation of precursor cells into progenitor cells (refs. *74,116*; Teerds et al., manuscript submitted). Because of the high doses of estradiol necessary to induce this effect, the physiological significance of estradiol in precursor cell differentiation remains to be further investigated (Fig. 8). Moreover, in vivo studies have demonstrated that prolonged exposure to estradiol in mice leads to the development of Leydig cell tumors *(198,199)*.

Leydig Cell Progenitor Proliferation and Differentiation

The newly formed Leydig cell progenitors, both in the (pre)pubertal testis and the adult testis after EDS treatment, have the capacity to undergo one or more cycles of cell replication. In vivo studies in (pre)pubertal rats have demonstrated that LH is important for this process. In vitro studies have shown that LH's effectiveness in stimulating Leydig cell progenitor proliferation is dependent on the relative amount of TGF-α, TGF-β, IGF-1, and possibly also IL-1β. LH requires TGF-α together with IGF-1 or IL-1β to stimulate growth, while the actions of these growth factors are abolished by TGF-β *(146)*. Consistent with the hypothesis that TGF-β inhibits cell proliferation, is the observation in the (pre)pubertal testis that TGF-β immunoreactivity diminishes rapidly from the progenitor cells at the onset of the wave of Leydig cell proliferation and the generation of Leydig cells enriched in TGF-α (refs. *149,168*; Fig. 8).

There are indications that the wave of Leydig cell progenitor proliferation in the (pre)pubertal testis and the adult testis after EDS are under the control of the same factors. The growth stimulating factors TGF-α, IGF-1, and IL-1β are also present in the adult testis. Moreover, the patterns of appearance of TGF-α immunoreactivity during Leydig cell regeneration following EDS administration and the (pre)pubertal testis have been shown to be identical *(149)*. Studies in (pre)pubertal as well as adult rats following EDS administration have shown that T_3 not only advances the differentiation of precursor cells but also stimulates Leydig cell progenitor proliferation *(26,56)*. On the other hand, indirect evidence suggests that MIS inhibits progenitor proliferation in the (pre)pubertal testis *(162)* as well as in the adult testis during Leydig cell regeneration after EDS administration (ref. *126*; Fig. 8). The differentiation of the progenitor cells becomes apparent by changes in steroidogenic enzyme activity. By the time the progenitors have differentiated into immature Leydig cells 5α-reductase activity has reached its maximum level both in the (pre)pubertal testis and adult testis after EDS (refs. *92,172*; Fig. 8). LH has been shown to be essential for progenitor differentiation in the (pre)pubertal testis as well as in the

adult testis after EDS administration. These effects of LH are possibly mediated and modulated by locally produced growth factors.

Immature Leydig Cell Proliferation and Differentiation

By day 35 postpartum in the (pre)pubertal testis or day 35 after EDS administration, most progenitor cells have differentiated into immature Leydig cells. The proliferative activity of these cells is much than that of progenitor cells but still elevated compared with the mature testis *(20,71)*. The assumption that the cells have not yet lost their proliferative capacity, is supported by the observation that when the animals are treated with LH/hCG, immature Leydig cell proliferation is stimulated (Fig. 8). Concomitantly, the levels of several enzymes such as 3β-HSD, $P450_{scc}$, and $P450_{17\,\alpha}$ hydroxylase increase. These enzymes play an important role in testosterone synthesis *(92,171,172)*. Furthermore, 11β-HSD types 1 and 2 levels *(178,179)* increase, whereas 5α-reductase levels decrease. Testosterone is now the major steroid secreted by the Leydig cells (Fig. 8).

Dynamics of Mature Adult-Type Leydig Cells

Finally, most of the immature Leydig cells differentiate into mature adult-type Leydig cells which become terminally differentiated. This transition is characterized by a significant increase in the average cell size and a furthermore increase in the capacity of these cells to synthesize testosterone. This transformation is observed both in the pubertal/adult rat around the age of 60 d and in the regenerating testis between days 50 and 60 after EDS administration *(7,20,21,71)*. Nevertheless, the growth factors that have been shown to stimulate progenitor proliferation remain present, suggesting that at this developmental stage there are also factors produced in the testis that inhibit the proliferation of the immature adult-type Leydig cells that do not become terminally differentiated. The proliferative arrest of these cells can be overcome by a dramatic rise in plasma LH/hCG levels. Hence, the adult-type Leydig cell population both in the normal adult testis and following completion of the regeneration process after EDS administration, is a semistable population of cells, consisting of terminally differentiated mature Leydig cells and immature Leydig cells that still exhibit a very low rate of proliferation under normal physiological conditions. Supra-physiological levels of LH/hCG can affect the dynamics of at least part of this cell population and stimulate proliferation (Fig. 8).

CONCLUSIONS

There appear to be many similarities and hardly any discrepancies in the regulation of the development of precursor cells into mature adult-type Leydig cells during (pre)puberty and in the adult rat following EDS administration. Hence, Leydig cell development in the adult testis after EDS administration can be used as a model for the investigation of factors involved in the regulation of postnatal adult-type Leydig cell development. Because in the EDS-treated rat the regenerating Leydig cells are the only somatic cell type that undergoes growth and differentiation, one can speculate that this is an even better model for the study of the regulation of adult-type Leydig cell development than the (pre)pubertal testis in which beside Leydig cells, other somatic cells are also undergoing differentiation.

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7 Aging and the Decline of Androgen Production

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SUMMARY

Human male aging is associated with progressive decreases in serum concentrations of testosterone, which are not in response to decreased circulating basal luteinizing hormone (LH) concentrations, suggesting that reduced testosterone results from a primary deficit at the gonadal rather than the hypothalamic-pituitary level. This also is true of Brown Norway rats, a strain that has become widely used for studies of Leydig cell aging. Age-related reduced testosterone was found to result from reduced Leydig cell steroidogenesiss, but not by their loss. This chapter deals with the cellular changes, which are associated with reduced testosterone, and their causes. Age-related reductions have been reported in Leydig cell LH receptor numbers, intracellular cyclic adenosine monophosphate (cAMP) formation, steroidogenic acute regulatory protein, peripheral benzodiazapine receptor, the conversion of cholesterol to pregnenolone within the mitochondria and the subsequent conversion of pregnenolone to progesterone, 17α-hydroxyprogesterone, androstenedione, and ultimately testosterone in the smooth endoplasmic reticulum. Culturing isolated Leydig cells with LH maintained high levels of testosterone production by young cells but did not restore old cells to "young" levels. In contrast, culturing old cells with dibutyryl cAMP restored testosterone production to high levels, suggesting a deficit in the signal transduction mechanism between the LH receptor and cAMP production. Long-term suppression of steroidogenesis, accomplished by administering exogenous testosterone to middle-aged rats, prevented the steroidogenic aging of the cells, suggesting that ultimately steroidogenesis itself might result in age-related reductions in steroidogenesis. As measured with lucigenin, reactive oxygen production by old Leydig cells was found to be significantly more than by young Leydig cells. Microarray and Northern blot analysis revealed that the expression of genes for Cu–Zn superoxide dismutase 1, Cu–Zn superoxide dismutase 2, catalase, and glutathione peroxidase, the products of which protect Leydig cells from oxidative stress, are reduced as the Leydig cells age, as does their activities and protein levels. Depletion of glutathione with buthionine sulfoximine resulted in reduced testosterone production by young adult Leydig cells. Incubation of the cells with vitamin E delayed reduced testosterone production. Taken together, the available data suggests that changes in reactive oxygen and thus, an altered redox environment in aging Leydig cells might cause the changes in Leydig cells that result in age-related reduced testosterone production, and that the reactive oxygen might derive, at least in part, from steroidogenesis itself.

Key Words: Aging; Leydig cell; LH; reactive oxygen; signal transduction.

INTRODUCTION

Aging even in healthy men typically is associated with progressive decreases in serum concentrations of testosterone (Fig. 1) *(1)*. Testosterone decline is associated with reduced sexual function, muscle function, and bone density *(2,3)*, and thus, has significant medical and public health consequences. Decreases in serum levels of testosterone also occur in rodents as they age *(4–10)*. The ability to experimentally manipulate rodent testes, and to isolate and culture rodent Leydig cells, make it possible to address the cellular and molecular changes in aging Leydig cells, which result in their reduced ability to produce testosterone.

REGULATION OF ADULT LEYDIG CELL FUNCTION

Overview

Adult Leydig cell testosterone production depends on the pulsatile secretion of luteinizing hormone (LH) by the pituitary gland into the peripheral circulation *(11,12)*. Acting predominantly through a cyclic adenosine monophosphate (cAMP)-dependent pathway, LH has both rapid (acute), and long-term (trophic) effects

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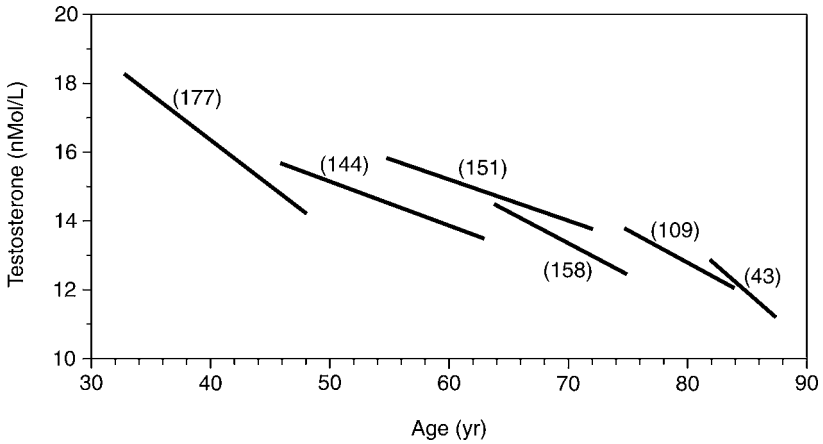


Fig. 1. Longitudinal effects of aging on serum testosterone concentrations in cohorts of men. (Redrawn from ref. *1*)

on Leydig cell testosterone production *(13,14)*. In its acute actions, LH binds to specific high affinity receptors on the Leydig cell plasma membrane, thereby initiating a cascade of events, illustrated in Fig. 2, which include LH receptor (LHR) coupling to guanine nucleotide-binding proteins, activation of adenylate cyclase, increased intracellular cAMP formation, cAMP-dependent phosphorylation of proteins through protein kinase A (PKA), and translocation of cholesterol to the inner mitochondrial membrane *(15,16)*. The conversion of cholesterol to pregnenolone is catalyzed by P450 side-chain cleavage enzyme (P450scc), located on the inner mitochondrial membrane. Pregnenolone moves out of the mitochondria to the smooth endoplasmic reticulum where it is converted to progesterone by 3β-(HSD) hydroxysteroid dehydrogenase enzyme. Progesterone is then acted upon by 17α-hydroxylase/C17-20 lyase (P450c17) to produce 17α-hydroxyprogesterone and then androstenedione. Finally, androstenedione is converted to testosterone by 17β-HSD (also referred to as 17-ketosteroid reductase) *(17,18)*. LH also has long-term (trophic) effects on steroidogenesis. In particular, numerous studies have shown that the major steroidogenic enzymes are regulated by LH through cAMP *(14,17,18)*.

cAMP Production

Intracellular cAMP concentration is a balance between the rates of cAMP synthesis and degradation, which are regulated by adenylyl cyclases and cyclic nucleotide phosphodiesterases, respectively *(16,19,20)*. The mechanism by which the binding of LH to its receptor ultimately produces cAMP has been studied extensively *(19–24)*. Binding of LH to the LHR, a seven transmembrane guanine nucleotide-binding (G) protein-coupled receptor, induces the binding of the cytosolic domain of the receptor to a G protein. In its resting state, the α-subunit of the trimeric G protein is bound to guanosine 5′-diphosphate (GDP). LHR binding to the G protein stimulates the exchange of bound GDP for guanosine 5′-triphosphate (GTP). This activates the α-subunit which then dissociates from the dimeric β–γ subunits of the G protein, and the active GTP-bound α-subunit then interacts with adenylyl cyclase to convert ATP to cAMP. The activity of the α-subunit is terminated by hydrolysis of the bound GTP by the action of the intrinsic GTPase activity of the α-subunit. The inactive α-subunit, now with GDP bound, reassociates with the β–γ complex, restarting the cycle. To date, about 20 distinct α-subunits have been cloned. These can be divided into four major subfamilies: adenylyl cyclase (Gs)-α, adenylyl cyclase inhibition (Gi)-α, Gq/11α, and G12α. There are G proteins, which are activators of Gs and those that are involved in Gi. At least nine closely related isoforms of adenylyl cyclases have been identified, and about 40 phosphodiesterases. Both the receptor and effector proteins involved in cAMP production are thought to be mobile within the plane of the membrane and so are influenced by the state of membrane fluidity. Consequently, changes in membrane fluidity could have significant consequences for cAMP production *(16,19,21,24,25)*.

The primary point of postreceptor control in the acute stimulation of steroidogenesis by LH is the conversion of cholesterol to pregnenolone on the inner mitochondrial membrane by P450scc *(26–28)*.The rate-limiting step in this process is the cAMP-dependent transport of cholesterol from intracellular sources into the mitochondria *(26–28)*. Protein phosphorylation through PKA is a key regulatory step in hormone-stimulated steroid formation

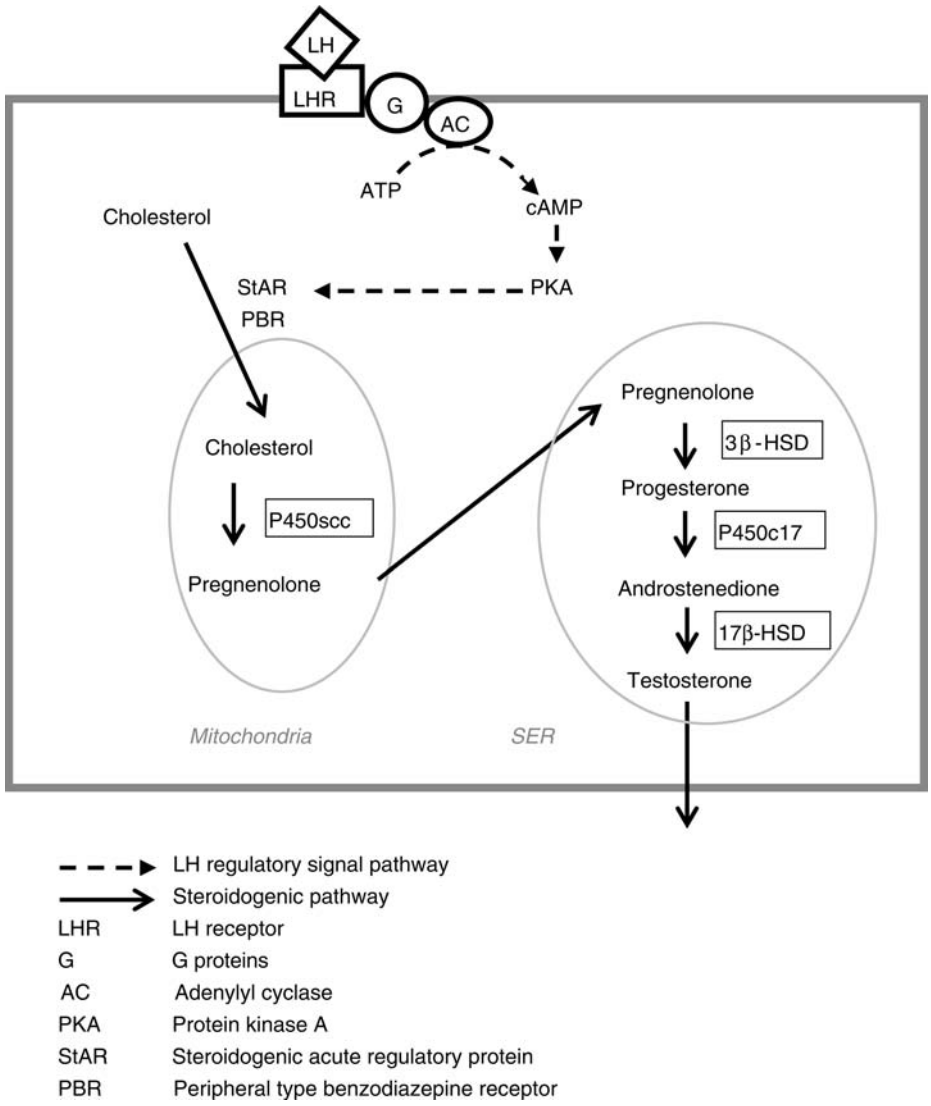


Fig. 2. Rat Leydig cell steroidogenesis.

(27,29,30). Maximal stimulation of cholesterol transport and steroid formation can occur at lower cAMP levels than can be induced by LH/human chorionic gonadotrophin (hCG); the concentration of hCG needed to induce maximal cAMP synthesis is at least 15 times higher than that needed for maximal testosterone production *(26,31)*. Although the compartmentalization of cAMP in the cells is the commonly proposed explanation for why relatively little cAMP is needed for maximal testosterone production under acute stimulation by LH *(32)*, the exact mechanism, including the amount and timing of cAMP dosage, remains uncertain.

Cholesterol Transport

A constant supply of cholesterol is needed as a substrate for steroid hormone synthesis in steroidogenic tissues. The immediate source of cholesterol in all steroidogenic cells is cholesteryl esters. There are two potential sources of cholesterol for steroidogenesis when the endogenous pool is reaching depletion, and they are *de novo* synthesis and exogenous lipoproteins *(33–35)*. Plasma lipoproteins are the major source of cholesterol for steroid productions in the adrenal gland and ovary, whereas Leydig cells produce most of their own cholesterol *(33–35)*. Scavenger receptor class B type 1 has been shown to be involved in the import of exogenous cholesterol from blood lipoproteins in rat Leydig cells *(33)*. Scavenger receptor class B type 1 is also regulated by LH, with hCG treatment resulting in dramatic increases in both messenger RNA and protein levels of SR-BI and increased uptake of high density lipoprotein cholesteryl esters *(33)*. Leydig cells also actively

synthesize cholesterol *de novo* from acetate. The rate-limiting enzyme in this synthetic process is 3-hydroxy-3-methylglutaryl-coenzyme A reductase. Usually, cholesterol in Leydig cells is stored in the form of either free cholesterol or cholesteryl esters. The cholesteryl esters can be hydrolyzed (mobilized) by cholesteryl esterases for steroidogenesis. Recent studies indicated that carboxyesterase (ES-10) and hormone sensitive lipase (HSL) plays significant roles in this mobilization process *(36–38)*. How these cholesterol synthetic and hydrolytic enzymes are regulated in Leydig cells is poorly understood.

Two proteins have been identified as having important roles in cholesterol transport across the mitochondrial membrane: peripheral-type benzodiazepine receptor (PBR) and steroidogenic acute regulatory protein (StAR) (Fig. 2). PBR is a high-affinity cholesterol-binding protein that is present in high levels in the outer mitochondrial membranes of steroid producing tissues. PBR takes up free cholesterol from a cytosolic donor and transfers it from the outer to the inner mitochondrial membrane *(39–41)*. Targeted disruption of the PBR gene *(42)* or treatment of cells with a PBR peptide antagonist *(43)* were found to inhibit cholesterol transport into mitochondria, and thus, to suppress steroid formation. Based on the knowledge of its structure, PBR has been proposed to function as a channel for cholesterol movement. Upon addition of hCG to Leydig cells, there is a rapid increase in PBR-ligand binding, which can be inhibited by a PKA inhibitor *(44,45)*.These observations suggest that cAMP-induced phosphorylation of PBR might be involved in hCG-stimulated steroidogenesis.

StAR is a 30 kDa protein that is processed from a 37 kDa mitochondrial signal sequence-containing precursor protein *(28,46,47)*. Evidence for StAR's important role in steroidogenesis comes in part from studies of congenital lipoid adrenal hyperplasia, an autosomal recessive disease in which synthesis of adrenal and gonadal steroids is severely impaired. This disease, which is characterized by minimal steroid production, has been reported to be the result of mutations in the StAR gene *(48)*. In addition, in response to trophic hormones, StAR synthesis has been shown to parallel steroid synthesis *(49,50)*, and transfection of StAR into COS-1 cells was shown to lead to an increase in steroid formation *(51)*.

There is evidence that PBR and StAR might function coordinately in the process of cholesterol transport *(52–54)*. When PBR and StAR were knocked out in MA-10 mouse Leydig tumor cells individually by antisense oligodeoxynucleotides, the ability of the cells to synthesize steroids in response to hCG was inhibited in both cases, suggesting that both StAR and PBR proteins are important elements of the steroidogenic machinery *(54)*. Recent studies have reported that StAR need not enter mitochondria to stimulate steroidogenesis, but rather might act at the outer mitochondrial membrane to initiate cholesterol transfer from the outer to the inner mitochondrial membranes *(52,53,55)*. Considering that PBR is localized to the outer mitochondrial membrane, it has been proposed that StAR is the initiator of cholesterol transport and that PBR is the "gate" for cholesterol entry into mitochondria *(54)*. One of the PBR-associated proteins (PAP7) can bind the regulatory subunit RI-α of PKA directly *(56)*. This macromolecular signaling complex (PKA–PAP7–PBR) might allow for local efficient catalytic activation and phosphorylation of StAR, leading to cholesterol transfer from the low affinity StAR to the high affinity PBR cholesterol binding protein *(57)*.

STEROIDOGENESIS IN AGING MEN

Decreases in plasma total and bioavailable testosterone concentrations have been reported in numerous studies of aging men, including studies in which particular care was taken to consider health and other possible confounding factors such as obesity, tobacco use, and alcohol use (Fig. 1; refs. *58–62*). The reported ages at which these decreases begin, and the extent of the decreases, differ among studies. For example, initial decrements were reported in men of age 33 yr in one study, but of age 50 yr in another *(62)*. Average annual decreases in serum testosterone levels between ages 40 and 70 have been reported to be as low as 0.4% *(60)* and as high as about 1.6% *(62,63)*. As testosterone clearance does not increase with age *(64,65)*, the lower serum levels of testosterone in elderly men suggest decreased Leydig cell testosterone production.

Most studies have reported that circulating basal LH concentrations increase somewhat with age in the human male *(58,60,61,66–74)*. This, in combination with reports of age-related decreased serum testosterone concentration, suggest a primary deficit at the gonadal rather than the hypothalamic-pituitary level. This interpretation is supported by the results of clinical studies showing that the ability of exogenously administered hCG to stimulate testosterone production is reduced in elderly as compared with young men *(66,68,75–78)*. In a series of recent studies, recombinant human (rh) LH has been delivered in pulses to young and elderly men whose endogenous LH was suppressed by the GnRH receptor antagonist ganirelix

(79–81). In these studies, the elderly men had significantly lower levels of serum testosterone than the young men despite similar serum levels of rhLH. Consistent with this, stimulation of gonadotropin secretion by short-term clomiphene citrate treatment of young and elderly men resulted in similar LH pulse frequency and amplitude, but lower serum testosterone levels in the elderly *(72)*. These results all point to a primary Leydig cell deficit in aging males. However, this does not rule out the possibility that changes in LH pulse frequency and/or amplitude also may contribute to reduced testosterone production *(82,83)*.

The blunted response to hCG and rhLH in elderly men might be explained by age-associated diminished Leydig cell numbers, the diminished capacity of Leydig cells to produce testosterone, or both. Most studies have reported that the number of Leydig cells in the human testis decreases with age *(84,85)*, but the data are difficult to interpret in part because the studies were not conducted with modern, unbiased counting procedures. Age-related changes in human Leydig cell structure, suggestive of altered cell function, also have been reported *(86)*. For example, Leydig cells of elderly men have been characterized as multinucleate and vacuolated, having abundant lipid droplets and intranuclear, or cytoplasmic crystalline inclusions, and with relatively little smooth endoplasmic reticulum, and relatively small mitochondria *(87)*. In elderly men with reduced serum testosterone, relatively more numbers of such cells were seen, suggesting that reduced serum testosterone may result from Leydig cell functional changes *(86)*.

Studies of testosterone and its precursors in fluids of the testes and in the spermatic vein have reported age-related reductions in the steroidogenic enzymes 3β-HSD, P450c17, and 17β-HSD *(88–91)*. Likewise, age-related decreases in 3β-HSD and P450c17 have been shown by incubating testicular tissue with testosterone precursors *(92,93)*. In a study of the serum concentrations of conjugated and unconjugated steroids in about 2400 men of ages 40–80, 17α-hydroxyprogesterone, androstenedione, and testosterone were reported to decrease with aging, whereas the level of progesterone did not change *(62)*, suggesting that the 17α-hydroxylase enzyme activity might be particularly susceptible to aging effects. However, a study of intratesticular steroids from men of 24–85 yr of age reported that the decline in Leydig cell function resulted from reduced pregnenolone formation in the mitochondria rather than from microsomal reactions that occur subsequent to pregnenolone formation *(94)*. From these studies, it seems clear that reduced testicular steroidogenesis with age might be a consequence of multiple changes in the steroidogenic pathway.

STEROIDOGENESIS AND LEYDIG CELL FUNCTION IN AGING RODENTS

Overview

Aging has been shown to result in reduced serum testosterone concentration in a number of species, including mice *(95–99)*, rats *(100,101)*, and birds *(102)*. Rat is focused in this chapter because this is the species from which most of our knowledge about Leydig cell aging derives. Depending upon the strain, rats typically live from 25 to 40 mo, with 3 mo-old rats generally, considered to be mature adults and rats of 20 mo or older considered to be aged. Age-related decline in serum testosterone has been reported for several rat strains *(4–10)*. In most strains, including Sprague-Dawley, Fischer 344, Long Evans, and Wistar, serum LH levels also have been shown to decline with aging *(6,7,10,103–105)*, which suggests that the reduced serum testosterone in these strains might be a secondary effect of hypothalamic-pituitary changes. This conclusion is consistent with the observation that the administration of LH to aged rats of these strains increased serum levels of testosterone *(105–107)*.

However, as indicated above, the age-related declines in serum testosterone levels in the human male are not correlated with decreased LH levels *(58,60,61,66–73)* and thus, are considered to result from *primary gonadal failure* rather than from changes in the hypothalamic–pituitary axis. In Brown Norway rats, as in the human, LH levels do not decrease significantly with aging, and serum FSH levels rise *(5,108,109)*. This similarity to the human is among the reasons that the Brown Norway strain has become widely used for studies of Leydig cell aging. Others are that these rats have a long life span and therefore, are amenable to experimental study even when they are old (the rats live to age 40 mo), and that they do not become obese and typically have no pituitary or Leydig cell tumors. These qualities make it possible to distinguish between age-related and confounding disease-related changes in this strain.

Leydig Cell Deficits

Most studies of aging rats have reported that the number of Leydig cells per testis was unchanged or somewhat increased compared with young controls *(9,108,110–112)*, suggesting that changes in the function of the Leydig cells, not their loss, accounts for reduced serum testosterone levels. In vitro studies have

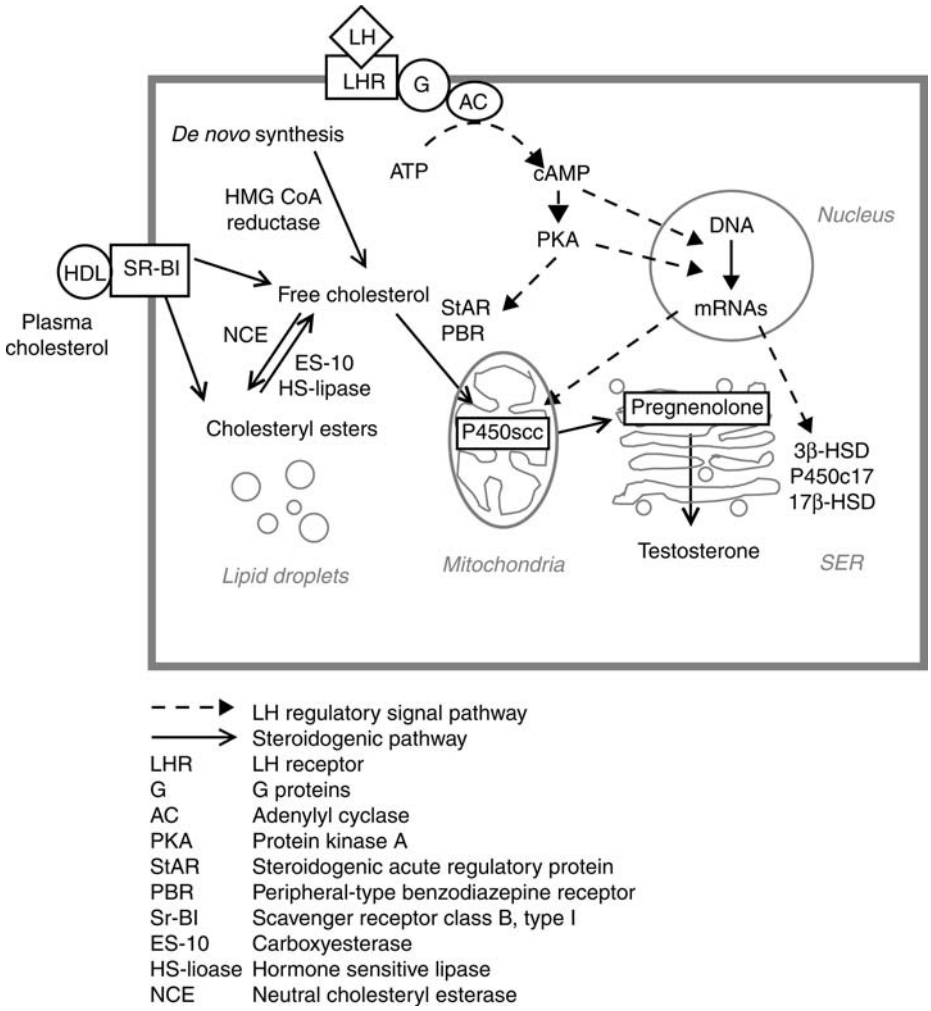


Fig. 3. LH regulatory and steroidogenic pathways in Leydig cells: locations of possible age-related defects that lead to reduced testosterone.

shown that the testes of aged Brown Norway rats or of Leydig cells isolated from the testes have a reduced capacity to produce testosterone compared to those of young adults *(9,108,113–116)*. As illustrated in Fig. 3, there are a number of changes in aging Leydig cells, which might explain their reduced ability to production testosterone. Binding studies with hCG revealed reduced receptor number in aged as compared with young Leydig cells *(117)*. In response to incubation with LH or hCG, cAMP production, and PKA activity were found to be reduced in Leydig cells from aged rats, indicating that signal transduction mechanism of these cells might be affected by aging *(117,118)*. Interestingly, although Leydig cells from young rats, which had received LH-suppressive testosterone-containing implants resemble old cells in their reduced volume, testosterone production and hCG-binding sites, incubation of these "young suppressed" cells with LH, resulted in cAMP production at the level of untreated young cells *(118)*. This suggests that reduced cAMP production by old cells might not be because of reduced LHR number, but rather to defects in the LH-cAMP signaling cascade, which reduce the responsiveness of the cells to LH stimulation. StAR and PBR protein and mRNA levels also were found to be reduced in Leydig cells from aged rats, suggestive of age-related deficits in cholesterol transport *(119–121)*. Additionally, the activities of each of P450scc, 3β-HSD, P450c17, and 17β-HSD were found to be reduced in Leydig cells from old rats *(120,122)*.

Using microarray technology, it is reported that a number of genes related to cholesterol metabolism and steroidogenesis, decreased with aging *(123)*. In addition to the steroidogenic enzymes *(120,122)*, these genes included scavenger receptor BI (SR-BI), ES-10, and HSL, all of which are involved in cholesterol

trafficking, and metabolism (Fig. 3; refs. *34,37,38*). As discussed earlier, SR-BI has been implicated as a major cell surface HDL receptor involved in cholesterol influx *(34,35)*, and ES-10 and HSL are involved in cholesterol mobilization. Down regulation of genes involved in cholesterol trafficking and metabolism suggests that the supply of cholesterol for steroidogenesis might be affected in aged Leydig cells. Indeed, in the Sprague Dawley rat strain, the ability of hCG to mobilize (hydrolyze) stored cholesteryl ester for testosterone production was shown to be significantly reduced (by 65–75%) in aged cells. This might result from the age-related decline in neutral cholesteryl esterases such as HSL *(116)*. In addition to the declines in cholesterol mobilization, the *de novo* synthesis of cholesterol also might be affected by aging. For example, the activity of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, the rate-limiting enzyme in cholesterol biosynthesis, was shown to be decreased by about 70% between age 5 and 18 mo *(116)*.

Another significant group of genes found to be down-regulated with aging are members of the family that scavenge and/or repair free radical-induced damage, including Cu–Zn superoxide dismutase (SOD1), microsomal glutathione-*S*-transferase, and glutathione-*S*-transferase *(123)*. The reduced expression of these genes could be of importance to Leydig cell aging because of the significant roles played by scavenging enzymes and glutathione in protecting cells from reactive oxygen species (ROS)-induced damage (*see* below).

It should be noted that the age-related changes in Leydig cells are not restricted to the proteins, which are directly involved in steroidogenesis. Relaxin-like factor/insulin-like factor 3, a circulating peptide hormone of the relaxin-insulin family, which is known to stimulate growth of the gubernaculum and to be involved in testis descent testis, is expressed constitutively in adult Leydig cells and is considered a good marker for the differentiation of Leydig cells *(124)*. Recently, it has been found that relaxin-like factor/insulin-like factor 3 mRNA and protein levels decreased significantly with age in rat Leydig cells *(125)*, suggesting that aging affects not only steroidogenesis but also other functions.

Luteinizing Hormone

The functional changes in aging Leydig cells, which lead to their reduced ability to produce testosterone might be caused by changes that occur outside the Leydig cells, which impinge upon them (extrinsic factors), or by changes within the Leydig cells themselves (intrinsic factors). The most obvious possible explanation is understimulation of aging Leydig cells by LH. Although serum LH levels do not change significantly with age *(108,109)*, age-related changes in LH pulse amplitude and frequency have been reported *(126,127)*, which could affect Leydig cell testosterone production. However, in experimental studies, neither in the vivo administration of exogenous LH to old rats *(128,129)* nor in the vitro culture of old cells with LH *(117)* was found to increase the ability of old Leydig cells to produce testosterone. These data do not rule out LH as contributing to reduced testosterone production, but strongly suggest that LH deficits are not the major cause.

Cyclic Adenosine Monophosphate

The ability of Leydig cells from aged rats to produce cAMP is reduced significantly *(117)*. Given the importance of cAMP in steroidogenesis, it was reasoned that reduced cAMP might cause the cellular changes, which lead to reduced testosterone production in aged cells. If this was the case, culturing the aged cells with dibutyryl cAMP (dbcAMP), a membrane-permeable cAMP agonist that bypasses the LHR-adenylyl cyclase cascade, should increase testosterone production by these cells. Indeed, culturing Leydig cells for 3 d with dbcAMP restored testosterone production by old Leydig cells to levels approximating those of young cells *(130)*. StAR and P450scc also were restored to young levels. These results, which demonstrated that bypassing signal transduction largely reverses the steroidogenic decline by the aged cells, strongly suggested that the reduced ability of old cells to transduce the signal between LH and cAMP production is largely responsible for reduced steroidogenesis by aged Leydig cells.

A number of studies designed to elucidate the possible causes of reduced cAMP by old Leydig cells have been reported *(117,130)*. The differences in cAMP production between young and old cells persisted when the old Leydig cells were cultured with LH and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine, suggesting that the decreased cAMP production in old cells resulted from changes in cAMP synthesis, not cAMP metabolism. LHRs are coupled to adenylyl cyclase through G proteins. Forskolin can activate adenylyl cyclase, thus, bypassing the hormone receptor–G protein signal transduction pathway. When stimulated with forskolin, old cells produced the same amount of cAMP as young cells, suggesting that adenylyl cyclase, and adenylyl cyclase–Gs protein interaction are maintained in the old cells. Pretreatment of old cells with pertussis toxin, which inhibits Gi proteins, did not restore the LH-stimulated cAMP production to the level of young cells, suggesting that changes in Gi protein are

unlikely to be the cause of old cells producing less cAMP than young cells. Pretreatment with cholera toxin, which bypasses the LHR and activates Gs proteins directly, increased the ability of old cells to produce cAMP almost to the levels of young cells, suggesting that Gs protein are well maintained in old cells as well. These observations pointed to defects in the coupling of the LHR to adenylyl cyclase through Gs proteins as the likely cause of reduced cAMP production.

Free Radicals and Redox Environment of Aging Cells

The mechanism by which coupling of the LHR to adenylyl cyclase becomes less efficient in aged Leydig cells is uncertain. The literature is replete with hypotheses to explain age-related changes in cells in general, including late-onset gene expression, telomere shortening, gene modifications, changes in the immune system, and accumulated reactive oxygen-induced damage to DNA, lipids and/or proteins *(131–136)*. Among these, the results of studies of Brown Norway rat Leydig cells are the most readily explained by free radical-induced damage, though the evidence for this is tenuous. The risk of damage from ROS probably is particularly high for steroidogenic cells. These cells, through the P450 enzymes, use molecular oxygen for steroid biosynthesis *(137)*, and there is evidence that this results in ROS production in addition to that produced by the mitochondrial electron transport chain *(138)*. Among the observations that support the hypothesis that free radical damage might be responsible for age-related functional deficits in Leydig cells are the following:

1. In vitro studies have shown that hydrogen peroxide can inhibit Leydig cell steroid production, perhaps by interfering with cAMP production or with cholesterol transport *(139–142)*. Related to this, incubation of kidney cells with hydrogen peroxide was found to uncouple the dopamine receptor from adenylyl cyclase-mediated cAMP synthesis in a PKC-dependent manner, suggesting that a redox shift to an oxidizing environment could be linked to uncoupling of the receptor-cyclase interaction *(143)*.
2. In many cell types including Leydig cells *(144)*, ROS production has been shown to increase with age.
3. Based on the hypothesis that, over time, reactive oxygen produced during Leydig cell steroidogenesis itself might contribute to the reduced steroidogenesis which occurs with aging, we demonstrated that the chronic suppression of steroidogenesis in Brown Norway rats prevented or delayed age-related reductions in Leydig cell function *(145)*.
4. Antioxidant enzymes are decreased with age in rat Leydig and adrenocortical cells *(146–148)*. In rat Leydig cells, the major enzymatic and nonenzymatic antioxidants, including SOD1, SOD2, glutathione peroxidase-1 (GPX-1), catalase (CAT), and reduced glutathione have been reported to decrease with age *(147,148)*.
5. Correlated with such decreases, the extent of lipid peroxidation in isolated Leydig cell membrane fractions was shown to be significantly elevated with age *(147)*. This is significant because lipid peroxidation can affect membrane structure and/or fluidity, and virtually every event associated with steroidogenesis is dependent on the integrity of cell membranes. Moreover, it has been shown in other cells that perturbation of membrane composition and/or fluidity can affect cAMP production *(149,150)*.
6. Long-term supplementation with the antioxidant vitamin E, delays age-related decreases in steroidogenesis, and longterm vitamin E deficiency enhances the decreases *(151,152)*.

These observations, taken together, support the hypothesis that reactive oxygen might play a significant role in the reduced ability of old Leydig cells to produce testosterone. However, most of the available data are descriptive and correlative; cause-and-effect relationships have yet to be proven.

Cyclooxygenase 2

LH, in addition to its role in stimulating cholesterol transport and the steroidogenic enzymes through cAMP, stimulates arachidonic acid release from Leydig cells through phospholipase A2 and/or acyl-CoA synthetase *(153–158)*. Arachidonic acid has been reported to be involved in modulation of the acute effects of LH on steroidogenesis *(153–158)*. Cyclooxygenase 2 (COX2) is an inducible enzyme involved in metabolizing arachidonic acid. In MA-10 Leydig tumor cells, inhibition of COX2 was shown to enhance the response of the cells to dbcAMP stimulation *(156)*. In Brown Norway rat Leyidg cells, COX2 mRNA levels increase with age *(159)*. In a recent study *(160)*, the incubation of aged Leydig cells with a COX2 inhibitor resulted in significantly increased testosterone biosynthesis, suggesting that increase in the metabolism of arachidonic acid by COX2 might play a role in age-related decline in testosterone biosynthesis. That too, was suggested by an in vivo study of old rats in which one month treatment with a COX2 inhibitor resulted in dose-dependent increases in both serum testosterone concentration and StAR levels *(160)*. Although, it has not been shown whether inhibition of COX2 also affects StAR and

testosterone production in young cells and/or young animals, the evidence suggests that changes in cAMP-COX2-StAR might be among those that are involved in the reduced testosterone production by old Leydig cells.

Estrogen

In aging human males, a dramatic increase in the ratio of free estradiol to free testosterone has been observed in the serum *(161)*. This is also true for aging Brown Norway rats *(162)*. P450c17 expression has been shown to be suppressed by exposure of Leydig cells to estrogen *(163)*. In addition,normally, the enzymes that, metabolize and/or detoxify estrogens decrease significantly with age in Leydig cells. For example, 17β-HSD-10, an enzyme, which converts estradiol to its inactive metabolite estrone, has been shown to decrease with aging *(161)*. Estrogen sulfotransferase, which catalyzes the sulfoconjugation and inactivation of estrogen, is highly expressed in Leydig cells *(164)*. Knockout of the estrogen sulfotransferase gene resulted in an age-dependent impairment in steroidogenesis of Leydig cells *(165)*. The deficiency was reversed by androstenedione but not progesterone supplementation, suggesting that the reduced activity of P450c17 was responsible for decreased steroidogenesis *(165)*.

Growth Hormone/Insulin-Like Growth Factor Axis

The effect of the growth hormone (GH)/insulin-like growth factor (IGF)-1 axis on Leydig cell function is well established *(166)*. There is good evidence that GH and IGF-1 decrease with aging in both human and rodents *(166,167)*. In men, 6–12 mo of GH treatment resulted in significant increases in plasma IGF-I levels and in hCG-stimulated testosterone concentration *(168)*. Because testosterone is known to affect the GH/IGF-1 axis, it was postulated that somatopause and gonadopause in aging men are linked, with hyposomatotropism responsible in part for reduced Leydig cell steroidogenesis, and reduced steroidogenesis, in turn, exacerbating the decline in GH/IGF1 *(167)*. Insulin, which shares many functions with IGF-1, has also been shown to be involved in the maintenance of Leydig cell steroidogenic function *(169)*. Serum testosterone concentration, and Leydig cell steroidogenic function are reduced significantly in patients with type 1 diabetes *(170)* and in spontaneous *(171)* or streptozotocin-induced *(172)* diabetes in animal models. In human males, insulin resistance has been shown to be associated with decreases in serum testosterone levels *(173)* and testosterone secretion from the testis *(170)*.

Cytokines and Nitric Oxide

In the interstitial compartment, macrophages are physically associated with Leydig cells. There is good evidence that two cell types are functionally related. Local inflammation and infection can activate macrophages to produce cytokines (e.g., interleukin-1, tumor necrosis factor-α), which have been shown to negatively regulate Leydig cell steroidogenesis *(174)*. Aging, in general, has been shown to be associated with increases in proinflammatory cytokines *(175)*. Although testicular macrophages change ultrastructurally with aging (e.g., accumulation of lipofuscin granules) *(176)*, their functions and intratesticular cytokine concentrations have not been defined in aging animals.

Macrophages also are major sources of ROS *(177)*. The possible contributions of ROS to age-related reductions in Leydig cell function have been discussed earlier. Recently, lipopolysaccharide endotoxemia has been shown to affect Leydig cell mitochondrial function and steroidogenesis by inducing ROS production from testicular macrophages *(178)*. Nitric oxide (NO), produced by macrophages, inhibits Leydig cell steroidogenesis *(179)*. Whether or not there is a prominent endogenous NO-generating system in adult Leydig cells is in dispute, but it is possible that aging affects this system *(180,181)*.

Extracellular Matrix

Extracellular matrix (ECM) accumulates in the interstitial compartment of the aging testis in close association with Leydig cells *(182)*. This is particularly evident in the regressed testes of aged Brown Norway rats (our unpublished results). In a microarray study of Brown Norway rat Leydig cells *(123)*, Age-related increases in two important protease inhibitors, tissue inhibitor of metalloproteinase 3 and plasminogen activator inhibitor 1 were detected. Tissue inhibitor of metalloproteinase and plasminogen activator inhibitor 1 inhibit endogenous proteolytic housekeeping activity, and play important roles in wound repair, ECM degradation, and fibrinolysis *(183)*. Their upregulation with age suggests that protease activities normally, responsible for ECM turnover might be suppressed in aged Leydig cells. This could result in the accumulation of ECM in the interstitial compartment of aging testes. Type IV collagen is an important component of ECM. It has been shown to affect progenitor Leydig cell proliferation *(184)* and in some *(185)* although not all *(186)* studies to inhibit steroidogenesis in adult Leydig cells. The accumulation of ECM in the interstitial compartment of aged rat testes might contribute to the reduced steroidogenesis by associated Leydig cells.

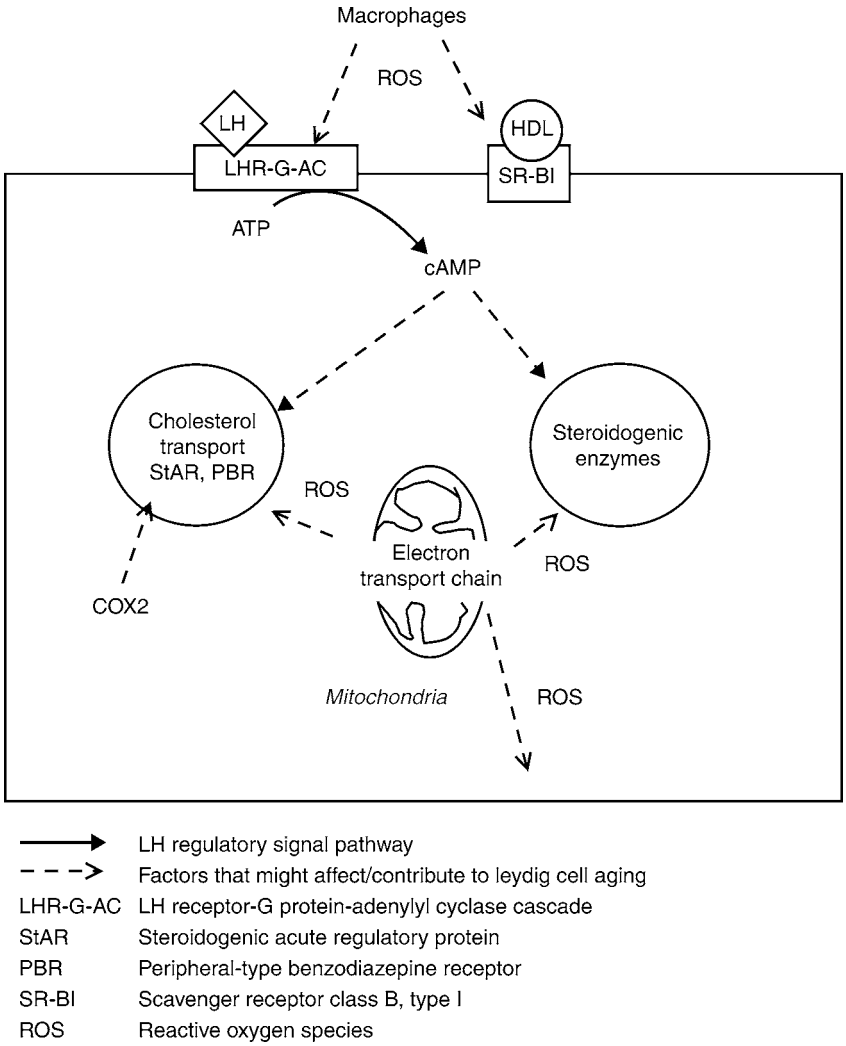


Fig. 4. Locations and possible sources of the defects in the steroidogenic pathway of aged Leydig cells.

CONCLUSION

Figure 4 summarizes the factors discussed in this chapter, which might contribute to Leydig cell aging. ROS derived from the mitochondrial electron transport chain, steroidogenesis and/or macrophages, by altering the redox environment of the aging Leydig cells *(187,188)*, might cause damage to Leydig cell membrane lipids and proteins, which, in turn, might result in the reduced LH signaling that characterizes aging Leydig cells. Reduced LH signaling would be expected to affect cAMP production, cholesterol transport through StAR and/PBR, and the steroidogenic enzymes. Age-dependent increases in COX2 also might contribute to reduced StAR and thus in reduced steroidogenesis. Additionally, other hormones and/or growth factors, including estrogen, GH/IGF-1, cytokines, NO, and ECM also might be involved in age-related reductions in Leydig cell steroidogenesis.

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III Steroidogenesis

8 Regulation of Leydig Cell Cholesterol Metabolism

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SUMMARY

Steroidogenic tissues have special requirements for cholesterol, which is used as a substrate for tissue specific steroid biosynthesis. Because of this, all steroidogenic tissues, including Leydig cells, have evolved multiple cholesterol delivery pathways and an efficient intracellular cholesterol transport system to ensure constant supply and adequate availability of cholesterol. There are four potential sources, which could contribute to the putative "cholesterol pool" needed for steroidogenesis: (a) *de novo* synthesized cholesterol, (b) stored cholesteryl esters, (c) exogenous lipoprotein-supplied cholesterol, and (d) plasma membrane-derived cholesterol. Among these, the cholesterol-rich plasma lipoproteins are often the most utilized source of cholesterol for steroid production. Cells acquire lipoprotein–cholesterol both by classic LDL receptor-mediated endocytosis and by selective uptake pathways. In the latter case, lipoprotein cholesteryl ester is selectively internalized without the concomitant uptake and lysosomal degradation of the entire lipoprotein particle. This bulk cholesterol delivery pathway is mediated by a scavenger receptor class B, type-I protein, which is highly expressed and hormonally regulated in steroidogenic cells. In addition to an adequate supply of intracellular cholesterol, steroidogenic cells also require efficient and controlled delivery of cholesterol to outer mitochondrial membranes, and subsequently, to inner mitochondrial membranes for P450scc catalyzed pregnenolone production—the precursor product for all steroids. Although, the exact steps involved in intracellular cholesterol transport to the outer mitochondrial membrane are not yet defined, it appears that vesicular/nonvesicular (through carrier protein) transport processes and interactions between mitochondria, and lipid droplets are probably involved. Two highly studied proteins, peripheral-type benzodiazepine receptor, and steroidogenic acute regulatory protein/steroidogenic acute regulatory protein D1, may function individually, or in concert, to subsequently facilitate the transfer of cholesterol from the outer to inner mitochondrial membranes—the rate-limiting step in steroidogenesis. The present chapter highlights the current understanding of these critical events in Leydig cells; i.e., the acquisiton, intracellular processing, transport, and utilization of cholesterol as the substrate for testosterone production.

Key Words: Cholesterol transport; cholesterol transport proteins; cholesteryl esters; endocytic pathway; HDL; LDL; LDL-receptor; lipid droplets; selective pathway; SR-BI; steroidogenesis.

INTRODUCTION

Testosterone, the principal secretory androgen product of the testis in most mammalian species, is produced almost exclusively by testicular Leydig cells *(1–6)*. It is biosynthesized from cholesterol in a series of biochemical reactions catalyzed by specific enzymes located in Leydig cell mitochondrial, and microsomal membranes *(7)*. Leydig cell testosterone steroidogenesis is primarily regulated by pituitary luteinizing hormone (LH) and, like other hormone-responsive steroidogenic tissues, testosterone production occurs in two distinct phases—an acute phase and a chronic phase. The *acute phase* of steroid hormone production is characterized by the rapid delivery of cholesterol to inner mitochondrial membrane (IMM) sites where the cholesterol side-chain cleavage enzyme, P450scc (also designated as CYP11A1) converts cholesterol to pregnenolone *(7–9)*. In contrast, *chronic* testosterone production is mediated mainly at the level of gene transcription, and leads slowly to an alteration in various steroidogenic enzymes, which either increase, or decrease the synthetic capacity of the Leydig cell *(10)*. Current evidence suggests that hormonal regulation in Leydig cells is, also, mediated by multiple signal cascades including cAMP-protein kinase A (PKA), serine/threonine AKT kinase (AKT, also called protein kinase B or PKB)- phosphatidylinositol 3-kinase (PI-3K),

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protein kinase C (PKC), mitogen-activated protein kinases (MAPKs), and intracellular Ca^{2+} signaling proteins *(11–13)*. In addition, other biologically active agents including growth factors, steroids, prostaglandins, and cytokines can influence the Leydig cell response through endocrine, autocrine, or paracrine regulation *(14–16)*.

It is now increasingly recognized that both the amount, and the efficient intracellular processing of the steroid precursor, cholesterol, is crucial for initiating and sustaining steroidogenesis *(9,17–21)*. There are several potential sources of cholesterol, which could contribute to the "cholesterol pool" needed for steroidogenesis in both adrenal and gonadal tissues. The cholesterol may be synthesized *de novo* in the endoplasmic reticulum (ER) *(22)*, or obtained directly from serum lipoproteins through "endocytic"*(17,18,23)* and/or "selective" pathways *(19,20,24)*. Cholesterol can also be supplied by the rapid hydrolysis of intracellular cholesteryl esters stored in the form of lipid droplets *(18,25)*; in some cultured cell systems, it can be derived from the plasma membrane (PM) as well *(26,27)*. Extensive work carried out in the past two decades on the adrenal, and ovary *(17–20)* has led to the clear demonstration that plasma lipoproteins are the major source of cholesterol for steroidogenesis in these tissues. In the testis, however, the story is more complex. Current evidence indicates that under normal physiological conditions, testicular Leydig cells rely heavily on endogenously produced cholesterol for testosterone production. However, under altered physiological conditions (e.g., gonadotropin-induced desensitization of the steroidogenic response) the additional demands for cholesterol are met by upregulation of lipoprotein–cholesterol delivery systems, most notably through the "selective" pathway and the use of exogenously supplied plasma lipoproteins *(19,20,28)*. In this chapter, the major pathways associated with the acquisition, and intracellular trafficking of cholesterol, emphasizing the relevance of these pathways to testosterone biosynthesis in Leydig cells were discussed.

HOW CELLS OBTAIN CHOLESTEROL

Information about Leydig cell cholesterol acquisition for testosterone synthesis is fragmentary, and complicated by apparent differences among species. Most of the studies involving biochemical measurements on intact animals were carried out on rodents (mainly rats), so it is these species, which provides the current working knowledge. In this section, initially the pathways available for cholesterol acquisition will be discussed, subsequently the situation as found in the rat will be discussed, and finally, Leydig cell cholesterol uptake in other species and various cell lines will be discussed. This information will be further summarized in Table 1.

Cholesterol Acquisition

De Novo Cholesterol Production

All cells and especially steroidogenic cells are capable of endogenous (*de novo*) synthesis of cholesterol. Cholesterol synthesis primarily occurs in the cell cytoplasm (specifically in the ER) from acetyl Coenzyme A (acetyl CoA) through a series of enzymatic reactions involving at least five distinct phases: (a) acetyl CoA units condense to form 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), (b) HMG-CoA is converted to mevalonate, (c) mevalonate is phosphorylated and converted to a 5-carbon isoprene unit called isopentenyl pyrophosphate (IPP), (d) IPP units condense to form the 30-carbon squalene unit, and (e) squalene cyclizes to lanosterol, which is further metabolized through a series of reactions to form cholesterol *(29,30)*. The enzyme HMG-CoA reductase, which catalyzes the conversion of HMG-CoA to mevalonate in step 2 aforementioned, is the rate-limiting step of cholesterol biosynthesis *(31–33)*, but is, also, subject to complex regulatory control involving four distinct mechanisms: feed-back inhibition *(31,33)*, gene expression through the regulated cleavage of the membrane associated sterol regulated element binding protein-2 (SREBP2) *(34)*, ubiquitination and proteolytic degradation *(33)*, and phosphorylation-dephosphorylation *(31,35)*.

De novo synthesized cholesterol may be used directly (or stored as free cholesterol [FC] in the PM and other cell membranes, or as cholesteryl ester in lipid droplets) for steroid synthesis or a variety of other cell uses. Such *de novo* synthesized cholesterol is readily available for steroid production but limited in quantity, so cells which have special needs often obtain additional cholesterol from exogenous sources.

Endocytic Pathway

In addition to their endogenous cholesterol synthesis, all steroidogenic cell types have some capacity to internalize cholesterol through the low-density lipoprotein (LDL) receptor-mediated endocytic pathway *(23)*. The LDL receptor is a transmembrane glycoprotein of 839 amino acids, which specifically binds apoB 100 (LDL), and apoE-containing lipoproteins *(23,36)*. Following binding, the LDL (or apoE lipoprotein) complex is recruited into clathrin-coated endocytic vesicles, and is rapidly internalized through the mediation

Table 1
Cholesterol Source and Leydig Cell Testosterone Production

Species	*Cell type*	*Comments*
A. *De novo* synthesized cholesterol		
Rat	Intact testis	Based on cholesterol feeding and tracer incorporation studies, Morris and Chaikoff *(58)* were first to suggest that testis utilize endogenous cholesterol source for steroid synthesis
	Intact testis	No effects of drug-induced hypocholesterolemia are observed either on CE or FC in intact testis *(59)*
	Leydig cells	Drug-induced hypocholesterolemia shows no stimulatory effect on HMG-CoA reductase activity in isolated Leydig cells *(31)* or reduction of cellular CE or FC levels *(28,61)*
Mouse	MA-10 cells	Depending on the experimental conditions MA-10 cells can utilize either *de novo* synthesized cholesterol, stored cholesteryl esters, LDL-cholesterol or PM cholesterol *(26,85)*
B. LDL receptor-mediated endocytic pathway		
Rat	Leydig cells	Basal Leydig cells express very low levels of LDLR protein, but levels are significantly induced following chronic treatment of animals with hCG *(28,62)*
	Leydig cells	hCG-desensitized rat *(28,60,68)* and mouse *(67)* Leydig cells, but not control cells, respond to in vitro addition of LDL with increases in testosterone production.
	Leydig cells	In vitro exposure of Leydig cells isolated from control rats to LDL shows no effect on testosterone production during acute hormonal stimulation, but LDL can provide cholesterol substrate and sustain steroidogenesis during prolonged hormone treatment *(68,69)*
Pig	Leydig cells	Cultured Leydig cells respond to LDL with increases in testosterone production both under basal condition and in response to hCG of cAMP treatment *(73)*
Human	Fetal Leydig cells	Show dependence on both LDL-supplied and *de novo* synthesized cholesterol as a precursor for testosterone synthesis
C. SR-BI-mediated selective pathway		
Rat	Intact testis	Infusion of HDL but not LDL to hyperstimulated and hypocholesterolemic rats leads to suppression of testicular *de novo* cholesterol synthesis, increased accumulation of CE and FC and elevated levels of plasma testosterone *(59)*. Such hCG-desensitized isolated Leydig cells respond to HDL with increased secretion of testosterone in vitro *(28,60,66)*
	Leydig cells	Leydig cells from control rats express very low levels of SR-BI and exhibit very little selective HDL-CE uptake measured both biochemically and morphologically *(28)*. In contrast, chronic hCG treatment is accompanied by a robust induction of SR-BI expression, alterations in cell surface architecture and increased selective CE uptake from [^{125}I]/[^{3}H]-HDL (biochemical) and BODIPY-HDL (morphological) ligands *(28)*
Rat	Leydig cell line	R_2C cells constitutively express high levels of SR-BI and selective pathway. cAMP addition shows no effect either on SR-BI or SR-BI-mediated selective HDL-CE uptake *(84)*

(Continued)

Table 1 *(Continued)*

Species	*Cell type*	*Comments*
Mouse	Leydig cell lines	MLTC-1 and MA-10 mouse Leydig tumor cells express only trace amounts of SR-BI protein under basal conditions, but SR-BI expression is enhanced significantly in response to cAMP. cAMP stimulation is also accompanied by increased selective HDL-CE uptake *(71,83,84)*
D. PM-derived cholesterol		
Mouse	Leydig cell line	Under certain conditions, stimulation of MA-10 cells with hCG or dibutyryl cyclic AMP can cause internalization of PM-cholesterol and its subsequent utilization for steroid synthesis *(26)*. Likewise, sphingomyelinase-induced disruption of PM cholesterol–sphingomyelin (SM) complex in mouse Leydig I-10 tumor cells results in increased translocation and utilization of released cholesterol for steroidogenesis *(27)*
Rat	Leydig cell line	R_2C cells which constitutively secrete high levels of steroids, also acquire part of the cholesterol substrate for steroidogenesis from the PM *(84)*

of a tyrosine-containing motif (NPXY) in the cytoplasmic tail of the receptor *(37)*. After endocytosis, the lipoprotein (apoB/E)-receptor complex enters the endosomal/lysosomal system, where the lipoprotein complex rapidly dissociates as the endosomal pH falls, and the protein receptor cycles back to the cell surface. The endosome containing whole apoB/E lipoprotein particles is then delivered to, and fuses with lysosomes, where the lipid and protein components are degraded by acid lipases and proteases. The FC released by the hydrolysis of cholesteryl esters by acid lipases enters the cytoplasm where it can now be utilized for product formation (e.g., steroids), membrane biogenesis, or transported to the PM, or to the ER for re-esterification, and subsequent storage in the lipid droplets.

Recently several proteins have been identified, which are involved in the trafficking of cholesterol in the LDL receptor (LDLR)-endocytic (endosomal/lysosomal) pathway. Among these, the Nieman-Pick C1 (NPC1) protein, a sterol-sensing protein, is a key participant in the LDL-derived cholesterol from the endosome/lysosome to the PM, or to the ER for cholesterol esterification *(38)*. Mutations in NPC1 lead to defective delivery of newly released LDL-cholesterol to the PM, impaired rates of cholesterol esterification, and aberrant accumulation of cholesterol, and other lipids in the late endosomal/lysosomal compartment *(39–41)*. Conversely, overexpression of NPC1 results in dose-dependent increases in delivery of endosomal cholesterol to the PM, and in esterification of LDL-derived cholesterol *(40)*. Two other proteins, NPC2, also known as HE1, a secreted sterol-binding glycoprotein, and MLN64, steroidogenic acute regulatory protein (StAR)-related lipid transfer domain protein, which can bind cholesterol, and promote its movement from donor to acceptor membranes, also participate in the movement of cholesterol out of lysosomes *(42)*. It has been proposed that MLN64 may be directly involved in steroidogenesis *(42,43)*.

Selective Pathway

Steroidogenic and liver tissues, also, obtain cholesterol from plasma lipoproteins such as high-density lipoprotein (HDL) by a unique process termed the "selective" cholesterol uptake pathway *(19,20,24,44)*. This pathway represents a major route for the delivery of cholesteryl esters (CE) to steroidogenic tissues of rodents, and human *(19,20,24,44)*, and differs from the classic endocytic LDLR pathway in that apoA1 containing HDL-CEs are taken into the cell without the concomitant uptake, and degradation of the entire HDL particle *(19,20)*. The selective pathway is described as a high capacity, physiologically regulated, bulk cholesterol transport system operating in steroidogenic tissues, and cells of a variety of species to deliver cholesterol for the synthesis of steroid hormones *(19,20)*, and in the liver to mediate the transfer of cholesterol into bile *(24)*. The selective pathway also operates in isolated hepatocytes, fibroblasts, adipocytes, and macrophages, although its function in these cell types is less clear *(19,20,24)*.

Scavenger receptor class B, type-I (SR-BI), approx 82 kDa glycoprotein, is a physiologically relevant cell surface receptor responsible for HDL-CE selective

uptake *(24)*. SR-BI is a member of the class B scavenger receptor family that also includes CD36, LIMPII, and SR-BII (an alternatively spliced form of SR-BI, in which 42 of the 45 *C*-terminal amino acid residues in the C-terminal cytoplasmic domain of SR-BI are replaced by 40 entirely different amino acid residues *[24,45]*). Tissues and cells, which express high levels of SR-BI, efficiently utilize the selective pathway deriving HDL-cholesteryl esters for use in steroid hormone, or product synthesis *(19,20,24,44)*.

It is also of interest that steroidogenic tissues, which express high levels of SR-BI in vivo (e.g., luteinized ovary and adrenal gland), are endowed with an intricate microvillar system for the trapping of lipoproteins. Closely apposed microvilli create channels, which distort the cell surface *(19,20,46,47)*, and it is the microvillar channels, where lipoproteins are trapped before the selective uptake of lipoprotein–CEs into cells *(19,20,47,48)*. Electron microscopic immunocytochemical techniques reveal heavy labeling for the HDL receptor protein, SR-BI, specifically in regions corresponding to microvilli and microvillar channels of adrenal and luteal tissues, and levels of SR-BI and the density and complexity of microvillar channels per cell surface show good functional correlation with selective HDL-CE uptake *(46,47)*.

Utilization of Plasma Membrane Cholesterol

In mammalian cells, FC representing 60–80% of total cellular cholesterol is localized in the PM, where it represents 35–45% of lipid molecules *(21,49)*. To what extent the PM cholesterol contributes to testosterone synthesis in primary Leydig cells remains to be established.

Cholesteryl Esters Stored in Lipid Droplets

Another source of cholesterol for Leydig cell steroidogenesis is cholesteryl ester stored in cytoplasmic lipid droplets. Irrespective of the source of cholesterol, whether synthesized *de novo* or derived from exogenous lipoproteins, steroidogenic cells tend to store certain amounts of cholesterol in its esterified form (CE) in lipid droplets as a reserved form of cholesterol for the synthesis, and maintenance of membranes and as a source of substrate for steroid hormone synthesis during acute hormonal stimulation *(18,25,50)*. CEs in cytoplasmic lipid droplets are produced by ER-localized acyl CoA:cholesterol acyltransferases that are allosterically activated by cholesterol *(51,52)*. Lipid droplets consist of a core of neutral lipid (triacylglycerol and/or and CEs) surrounded by a surface monolayer made up of polar lipids (cholesterol, phospholipids, and fatty acids) originally derived from the ER. The surface of the droplet is covered with a specific set of proteins called PAT (perilipin, adipophilin [also known as adipose differentiation-related protein or ADRP], and TIP47). This family of proteins *(53)* includes three spliced variants of perilipin (perilipin A, B, and C) and adipophilin, TIP47, a related protein, S3-12 *(53–55)*. Perilipins A and C and adipophilin, preferentially coat the lipid droplets of steroidogenic cells *(53,56)*. Despite their association with the lipid droplets, the PAT proteins seem to lack any catalytic domains and hence, it is felt that partner proteins in cells help with the putative functions of the PAT proteins in the steroidogenic cells. The only function that is currently known for any PAT protein is the perilipin A regulation of lipolysis in adipocytes; perilipin A restricts the access of hormone-sensitive lipase (HSL) and other lipases to the adipocyte lipid droplets under basal conditions, but facilitates lipase access to the droplet under lipolytically stimulating conditions (for review, *see* ref. *58*). As will be discussed in "cholesterol acquisition in other species, other conditions, and cell lines" section, normal Leydig cells from different species contain vastly different numbers of lipid droplets (rat Leydig cells typically with the fewest), yet all species are equally efficient in secreting testosterone.

Rat Leydig Cell Cholesterol Acquisiton

Morris and Chaikoff in 1959 were the first to examine the cholesterol requirements of the testis, and through a series of experiments in rats reported no suppression of testicular endogenous (*de novo*) cholesterol synthesis after feeding the animals diets rich in cholesterol *(58)*. These data were interpreted to suggest that the majority of the testicular cholesterol needed for steroidogenesis was derived from an endogenous source. Much later, Andersen and Dietschy *(59)* compared the effects of drug-induced hypocholesterolemia 4-aminopyrazolo-[3,4 D]-pyrimidine (4-APP) on sterol levels and cholesterol biosynthesis in three different steroidogenic tissues of the rat. Their results demonstrated that the steroidogenic cells of adrenals and ovaries of hypocholesterolemic rats had reduced levels of stored cholesterol esters and unesterified cholesterol, whereas showing enhanced organ cholesterol biosynthesis (i.e., a 42-fold increase in the adrenal gland, and a 2.7-fold increase in the ovary). However, the testes were not affected. These studies were further complimented by the demonstration that in rat-derived Leydig cells, HMG-CoA reductase activity (the rate limiting enzyme in cholesterol biosynthesis) was insensitive to 4-APP-induced hypercholesterolemia *(60)*. In a more

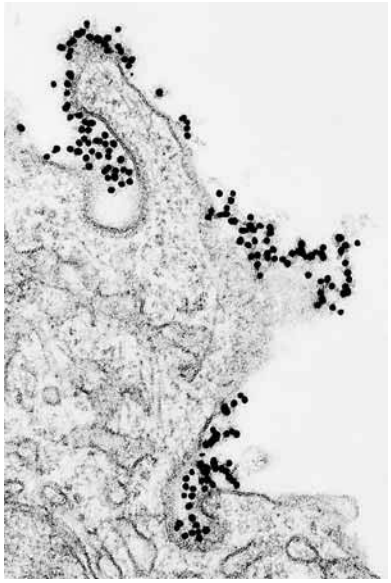


Fig. 1. Standard electron micrograph showing binding of prepared human LDL-colloidal gold complexes to the surface of a Leydig cell derived from an hCG-desensitized rat. The lipoprotein–colloidal gold complexes are shown on the cell surface and packed into clathrin coated pits associated with the B/E receptor endocytic pathway. Although not shown on this high magnification photo, gold particles are also found in endosomes and lysosomes within the Leydig cell cytoplasm *(28)*.

recent biochemical study performed by Azhar and colleagues *(28)*, it also became clear that isolated Leydig cells from rats treated with 4-APP showed no changes in CE or FC content. Indeed, the in vitro addition of LDL or HDL to these cells did not alter testosterone production—further demonstrating that these Leydig cells utilize endogenously synthesized cholesterol in preference to lipoprotein-derived cholesterol, and its esters under normal physiological conditions *(28,61,62)*. Similar results were reported for mouse Leydig cells *(4)*, cultured MA-10 mouse Leydig tumor cells *(63)*, and transplantable M5480P murine Leydig tumor cells following their harvest from mice *(64)*. In all, there seems to be a consensus that normal rodent Leydig cells preferentially utilize endogenously supplied cholesterol, and that short-term exposure to lipoproteins (LDL or HDL) appears to have no significant impact on androgen synthesis in these cells.

Only limited information is available about the functional expression of the LDL (B/E) receptor endocytic pathway in Leydig cells *(19,20)*. Normal rat Leydig cells express insignificant amount of the LDLR protein, but chronic treatment with human choriongonadotropin (hCG) leads to modest increases in LDLR levels *(28,65)*, increased binding to structures associated with the endocytic pathway (Fig.1) and increased function. During chronic gonadotropin (LH/hCG) treatment, which is also accompanied by desensitization of the steroidogenic response, several studies indicate that the extent of the testosterone production could be restored toward the control level by incubation of Leydig cells with cholesterol rich lipoproteins. This suggests that *de novo* cholesterol biosynthesis may be unable to replenish the depleted cholesterol substrate and sustain steroidogenesis. In one study, Quinn et al. *(66)* reported that the addition of human LDL to an incubation medium increased testosterone biosynthesis by Leydig cells from rats pretreated with a desensitizing dose of hCG, although it had no effect on testosterone production by cells from control animals. Charreau et al. *(60)* and Schumacher et al. *(67)* reported very similar findings for hCG-desensitized rat and mouse Leydig cells, respectively. Using an alternative strategy to deplete intracellular cholesterol, several investigators reported that the presence of LDL had little or no effect on the amount of steroid products synthesized during the acute phase of stimulation of rat Leydig cells (and MA-10 cells), respectively, but that it highly enhanced steroid secretion during prolonged hormonal stimulation by directly providing cholesterol substrate to cells *(53,68,69)*. A number of in vivo and in vitro studies suggest that under altered physiological conditions (e.g., chronic gonadotropin stimulation), rodent Leydig cells can take up and utilize HDL-derived CE for testosterone production. Andersen and Dietschy *(59)* were the first to report that in rats pretreated with both hCG and 4-APP (to simultaneously enhance Leydig cell steroidogenesis and decrease endogenous plasma lipoprotein cholesterol levels), infusion of hHDL, but *not* hLDL, suppressed the *de novo* cholesterol synthesis, promoted the accumulation of testicular FC, and esterified CE, and exhibited increased circulating levels of testosterone.

These latter results were interpreted to suggest that under the conditions of chronic gonadotropin treatment (desensitization), HDL is the major modulator of testicular cholesterol synthesis in rat Leydig cells, and preferentially contributes to cellular sterol levels. Quinn et al. *(66)* and Charreau et al. *(60)* further extended these observations when they reported that purified Leydig cells isolated from rats treated with a desensitizing dose of hCG respond to exogenously added hHDL with increased testosterone production. Subsequently, the presence of specific HDL binding sites was confirmed in rat testicular tissue *(70)*. Interestingly, treatment of rats for 4 d consecutively with a high dose of hCG shows a twofold increase in the number of membrane HDL binding sites, although apparent affinity remains unchanged *(71)*.

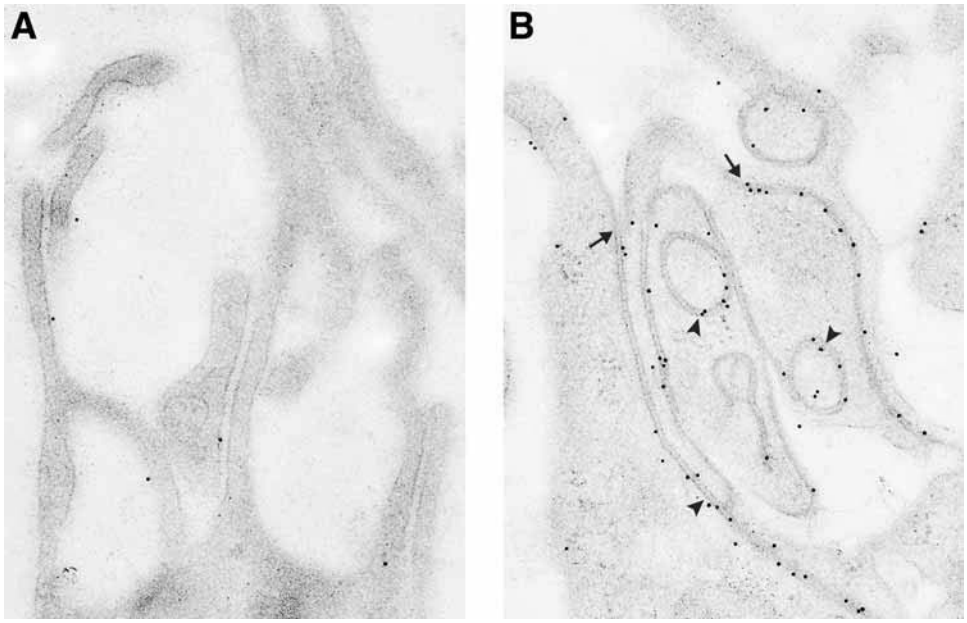


Fig. 2. Immuno-electron microscopic localization of SR-BI on the cell surface of control and hCG-desensitized rat Leydig cells. The control Leydig cell in Fig. 2A shows only an occasional cell surface gold particle representing gold-labeled antibody to SR-BI. In contrast, the Leydig cell from an hCG-desensitized animal (Fig. 2B) shows extensive gold labeling of SR-BI originating at the cell surface (*see* arrows) and extending into the peripheral cytoplasm of the cell in the form of double membraned linear and circular channels (*see* arrowheads). The Leydig cells of this preparation were immunostained with rabbit antipeptide SR-BI IgG followed by goat antirabbit IgG gold (10 nm) (*see also* ref. *30*).

Recently, the functional interactions between the HDL receptor (SR-BI), selective HDL-CE uptake, and testosterone production in Leydig cells of control, 4-APP-treated hypocholesterolemic and gonadotropin (hCG)-desensitized rats *(28)* were examined. As expected, Leydig cells from control rats exhibited no significant incorporation of HDL-derived radiolabeled CE into testosterone, poor selective HDL-CE uptake, few microvilli, microvillar channels, and low levels of SR-BI receptor protein. Drug-induced exaggerated hypocholesterolemia had no demonstrable effect on testosterone synthesis or HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis *(28)*. To examine the functioning of the SR-BI/selective pathway in response to gonadotropin-induced desensitization of Leydig cells, rats were treated with desensitizing doses of hCG. In agreement with earlier studies *(65)*, such chronic hCG treatment of rats greatly enhanced the expression of SR-BI in Leydig cells *(28)*. When isolated Leydig cells from the desensitized animals were incubated with either [^{125}I] DLT-[^{3}H] COE-HDL or BODIPY-CE-HDL, the increases in selective HDL-CE uptake was highly correlated with a corresponding increase in SR-BI levels *(28)*. It is noteworthy that although desensitized Leydig cells secreted low levels of testosterone, the rate of conversion of HDL-derived selective [^{3}H] cholesterol oleate $\rightarrow$ [^{3}H] testosterone was increased approx 10-fold *(28)*.

Whereas, Leydig cells from normal mature rats show little immunogold labeling for SR-BI at the electron microscope level (Fig. 2A), Leydig cells of hCG-desensitized (4 d hCG treatment) rats *(28)* show increased immunolabeling for SR-BI (Fig. 2B). Indeed, such Leydig cells exhibit a complex network of channels originating at the cell surface and coursing through the peripheral cytoplasm of the cells; generally these channels are outlined with immunogold representing sites of SR-BI localization (Fig. 2B) and, in fortuitous sections viewed at high magnification, it is often possible to identify intact HDL *(28)*. The dimeric/oligomeric forms of SR-BI in mouse and rat Leydig cells were examined. Control rat Leydig cells express higher levels of SR-BI monomers than do control mouse Leydig cells. However, both species show increased expression of SR-BI monomers and SR-BI dimers in response to chronic gonadotropin treatment *(71)*. Indeed, evidence suggests that SR-BI dimer formation is functionally linked to the selective CE uptake process *(71)*.

Thus, despite the preference of normal rat Leydig cells for endogenous production of the cholesterol needed for testosterone production, the cells retain the

Table 2
Lipid Droplet Content of Leydig Cells
From Various Mammalian Species

Species	*Volume density*
Rat	0.3 ± 0.1
Hamster	0.6 ± 0.1
Rabbit	3.8 ± 0.4
Monkey	5
Mouse	5.1
Guinea pig	7.9 ± 0.7
Aging humans	16.7
Dog	20.5 ± 1.3

Modified from refs. *1,2,76,77.*

capacity to upregulate their cholesterol reserves in case of need. They do this by utilizing both the endocytic, and especially the selective cholesterol uptake pathway to bring in lipoprotein cholesterol, which is either used directly for steroidogenesis, or is stored in lipid droplets as cholesteryl esters.

Cholesterol Acquisition in Other Species, Other Conditions, and Cell Lines

In contrast to rodent Leydig cells, isolated pig Leydig cells have been said to acquire more than 75% of their cholesterol for steroidogenesis from circulating lipoproteins *(72)*. Moreover, it was demonstrated that the addition of either human LDL or porcine LDL substantially enhanced both basal and hCG-stimulated testosterone production by pig Leydig cells *(72)*, and that LDL binding and internalization was also enhanced by hCG treatment *(73)*. Human fetal Leydig cells show similar dependence on both LDL-cholesterol and *de novo* synthesized cholesterol as a precursor for testosterone synthesis *(74)*.

Over the years, the occurrence and cellular levels of Leydig cell lipid droplets have been examined in various mammalian species both under normal and varying pathophysiological conditions. These investigations have yielded some interesting information. For example, as presented in Table 2, rat Leydig cells contain negligible levels of lipid droplets, whereas dog Leydig cells contain high numbers of lipid droplets followed by guinea pig, monkey, mouse, rabbit, and hamster. Bovine Leydig cells also express insignificant levels of lipid droplets *(75)*. Given that all these animals efficiently secrete testosterone suggests that Leydig cells utilize various mechanisms to satisfy their cholesterol needs for steroidogenesis. In other studies, aging has been shown to increase the CE content and expression of lipid droplets in both rat *(76)* and human Leydig cells *(77)*. Similar findings were reported for rat Leydig cells following chronic treatment of mature animals with hCG *(78)*. Studies involving genetic and experimental models suggest that testicular feminization is accompanied by a significant reduction in steroidogenesis along with the increased lipid accumulation and elevated levels of lipid droplets in Leydig cells *(79–81)*. Finally, excessive exposure of rats to heat leads to altered Leydig cell function and morphology including increased accumulation of lipid droplets *(82)*.

Unlike isolated Leydig cells from control animals, some Leydig cell *lines* respond to hormonal stimulation with an increased functional expression of SR-BI and the selective pathway *(71,83,84)*. Both MLTC-1 and MA-10 Leydig cells express only trace amount of immunodetectable SR-BI protein under basal conditions. However, cAMP analog stimulation of these two cell lines results in a robust induction of SR-BI (15- to 20-fold), increased selective HDL-CE uptake, and enhanced rates of steroid production *(71,83)*. Likewise, cells of the R_2C tumor line, which demonstrate constitutive steroidogenesis, also, exhibit an extremely high capacity for selectively-derived HDL-CE, and express far more SR-BI than the cAMP-induced maximal expression of SR-BI in MLTC-1 or MA-10 cells *(84)*. However, R_2C cells do not respond to cAMP treatment; no changes in either the levels of SR-BI or the extent of steroid production were noted *(71,84)*. Finally, all three cell lines, as well as primary Leydig cells, express only negligible amounts of the SR-BI isoform, SR-BII, and the expression of this isoform is unaffected by gonadotropin or cAMP treatment *(28,71)*.

Previous studies carried out by Freeman's laboratory *(26,85)* (and references therein) have indicated that Leydig cell lines under certain conditions can utilize the PM-pool of FC for steroid biosynthesis. It was reported that short-term stimulation of steroidogenesis in MA-10 cells with hCG or dibutyryl cAMP leads to significant decreases in PM cholesterol content, suggesting that PM-associated FC is preferentially utilized for steroid production under these conditions. Moreover, it was demonstrated that steroidogenic stimulation *per se* causes internalization of PM cholesterol and its utilization by the steroid biosynthetic pathway. Interestingly, the hormone-initiated loss of PM cholesterol content was replenished following incubation of cells with cholesterol-rich lipoproteins *(26)*. Similar results were reported for rat Leydig R_2C tumor cells, which constitutively secrete high levels of steroids *(85)*. Using an alternate strategy, Pörn et al. *(27)* demonstrated that disruption of the PM cholesterol–sphingomyelin (SM) complex by treatment

of mouse Leydig I10 tumor cells with sphingomyelinase results in increased translocation and utilization of PM cholesterol for steroidogenesis.

In addition to gonadotropin (LH/hCG) and its second messenger (cAMP), it appears that estrogen can independently regulate selective CE uptake and SR-BI expression in Leydig cells. A recent study has shown that Leydig cells of mice gene-ablated for estrogen sulfotransferase, a cytosolic enzyme that catalyzes the sulfoconjugation and inactivation of estrogens, and is expressed abundantly in the mammalian testis, accumulate excessive amount of CE with advancing age or in response to an exogenous estrogen challenge *(86)*. These changes in estrogen sulfotransferase derived Leydig cells are correlated with induced expression of SR-BI, and the ability of the cells to avidly internalize HDL-derived CE *(86)*. Taken together, these observations suggest that estrogen directly influences Leydig cell cholesterol homeostasis by impacting the functional expression of SR-BI and its associated selective CE uptake pathway.

Finally, some SR-BI transfected nonsteroidogenic cell lines (e.g., HEK-293 cells) *(71)* show characteristics similar to Leydig cells obtained from hormone desensitized rodents, in which the cells develop intracellular channels expressing SR-BI and conduct selective CE uptake.

INTRACELLULAR CHOLESTEROL TRAFFICKING

Trophic hormone-induced steroid biosynthesis is initiated with the transfer of cholesterol from intracellular sources into mitochondria. The first committed step in steroidogenesis is the conversion of cholesterol to pregnenolone, carried out by cytochrome P450 side chain cleavage (P450scc) enzymes (also termed CYP11A1), localized in IMMs *(7–9)*. Pregnenolone is subsequently metabolized in the mitochondria (and/or in ER) to other steroid products in a tissue specific manner. As in many metabolic pathways, this first step is the site of trophic hormone regulation, and its rate determines flux through the pathway. Of interest is the fact that the rate-limiting nature of this step is not determined by the *activity* of P450scc (and the conversion of cholesterol to pregnenolone), but rather by the delivery of cholesterol to the appropriate substrate site of P450scc (i.e., the translocation of cholesterol from the outer to inner mitochondrial site) *(8,9,11,12,18)*. Trophic hormones rapidly stimulate this process by acting through a cAMP messenger system, to facilitate the mobilization of intracellular cholesterol. The entire process of intracellular cholesterol transport to the mitochondrial P450scc site can be broadly divided into two separate but equally important steps: (a) mobilization of intracellular cholesterol and its transport to the outer mitochondrial membrane (OMM); and (b) translocation of OMM cholesterol to IMM sites (*see* Chapter 9).

Factors Directing the Mobilization of Cholesterol to Mitochondrial Outer Membranes

The mechanisms by which intracellular cholesterol is transported to mitochondria, are poorly understood at present, but may involve aqueous diffusion, vesicular transport, nonvesicular carrier-mediated transport *(8,49,87,88)*, and probably depend ultimately on facilitators such as transport proteins and cytoskeletal elements *(89–92)*. It is not yet clear whether any specific cholesterol transport factor is dominant, or unusual, for intracellular cholesterol transport in Leydig cells as compared to other steroidogenic tissues.

In the first step of an acute steroidogenic response to trophic hormones, activation of a neutral cholesteryl ester hydrolase (nCEH or cholesteryl esterase) by protein kinase-A (PKA), leads to the hydrolysis of CEs to FC. The FC is subsequently transported to outer mitochondrial membranes. Although, all the steps involved in nCEH mobilization of CEs in steroidogenic tissues have not been worked out in detail, considerable information is available for an analogous enzyme HSL, the major lipolytic enzyme in adipocytes *(25)*. When energy is required, hormone stimulation of β-adrenergic receptors on the PM of adipocytes triggers PKA-mediated phosphorylation of HSL and its translocation to lipid droplets where it catalyzes the hydrolysis of stored triglycerides. The activation of lipolysis is tightly linked to PKA-mediated phosphorylation of a protein, perilipin A. Under basal conditions, perilipin A is believed to function as a protective coat *(57)*, covering the lipid droplet and restricting the access of HSL to the lipid droplet. However, in response to lipolytically stimulating conditions, PKA-phosphorylated perilipin A undergoes a conformational change, facilitating the HSL access to the lipid droplet *(57)*.

It should be emphasized, that besides nCEH-mediated mobilization of lipid droplet-associated CE, or CE obtained directly from circulating HDL, the cAMP-PKA signaling cascade may also directly mobilize FC from the PM or other cellular membranes to the outer mitochondrial membrane.

As cholesterol shows poor solubility in water, aqueous diffusion alone is unlikely to contribute significantly to

sterol delivery to mitochondria. On the other hand, there is considerable evidence, obtained primarily through the use of pharmacological inhibitors, which at least a fraction of the cholesterol destined for steroidogenesis is shuttled to mitochondria through cholesterol-rich transport vesicles or tubules. Such intracellular vesicular traffic typically requires an integrated network of cytoskeletal elements (i.e., microfilaments, intermediate filaments, microtubules) that provide the tracks along which vesicles move, and an energy source (ATP) for motor proteins *(87,93–95)*. In addition, because cholesterol can desorb at a significant rate from intracellular membranes and lipid depots, and cells have many carrier proteins, carrier-mediated diffusion can also supply cholesterol to mitochondria. These diffusible carrier proteins could consist of cytosolic proteins with low affinity and specificity for cholesterol such as Sterol carrier protein$_2$ (SCP$_2$) *(8,91)*, or alternatively a family of newly described high-affinity lipid and cholesterol carriers *(95–99)*, whose lipid/sterol binding domains are referred to as StAR-related lipid transfer StAR-related lipid transfer domains (*see* cholesterol traffic to inner mitochondria membranes) could also play a role in cholesterol transport to mitochondria.

A number of studies indicate that pharmacological disruption of cytoskeletal structure–function in Leydig cells impacts intracellular cholesterol trafficking *(100–102)*. Disturbance of microtubule function through the use of colchicine, vinblastine, podophyllotoxine, taxol, and/or D_2O has been shown to interfere with both basal and LH-or cAMP steroid production in some Leydig cell preparations *(103,104)*. Also, treatment of rat Leydig cells with cytochalasins, which cause depolymerization of microfilaments, inhibit hormone- or cAMP-stimulated testosterone synthesis *(105,106)* presumably preventing the transport of cholesterol to mitochondria *(89,106,107)*. Finally, intermediate filaments especially of vimentin type may be important for intracellular cholesterol trafficking in the regulation of Leydig cell steroidogenesis *(91,108–111)* as morphological evidence provided by Russell and others *(110,111)* has shown vimentin to be abundant in Leydig cells and especially associated with mitochondria and lipid droplets—structures involved in cholesterol mobilization and testosterone production.

Nonvesicular transport mechanisms are also important for intracellular cholesterol homeostasis and steroidogenesis in Leydig cells. Nonvesicular transport can be mediated by both specific and nonspecific diffusible carrier proteins, which have hydrophobic pockets to trap cholesterol, and transport it across the aqueous cytosol. SCP$_2$ is the most studied cholesterol transfer protein in steroidogenic cells including Leydig cells. SCP$_2$ (also called nonspecific lipid-transfer protein; [nsL-TP]) is a 13.2 kDa basic protein that facilitates cholesterol, phospholipids and glycolipids transfer/exchange between the membranes and appears to play a key role in intracellular lipid trafficking *(92,112,113)*. By enhancing sterol transfer, it can stimulate enzymatic steps involved in cholesterol biosynthesis and esterification *(112,113)* and the mitochondrial conversion of cholesterol to steroid hormones *(114–116)* and bile acids *(112,116,117)*. Using Leydig tumor cell mitochondria, it has been demonstrated that purified SCP$_2$ (or a Leydig cell cytosolic fraction containing SCP$_2$) stimulates mitochondrial utilization of membrane cholesterol for cholesterol synthesis *(116)*; this stimulating effect can be abolished by pretreatment of the SCP$_2$ or Leydig cell cytosol with anti-SCP$_2$ IgG *(116)*.

Western blot analysis demonstrate high levels of SCP$_2$ expression in Leydig and Sertoli cells whereas it can not be detected in germ cells *(118)*. SCP$_2$ is localized in the highest concentration in peroxisomes, although, significant quantities of SCP$_2$ are also localized in ER, mitochondria and cytosolic fractions of Leydig cells *(118,119)*. In Leydig cells, SCP$_2$ expression is under trophic hormone (LH) stimulation. Acute stimulation with LH causes a rapid, but transient, increase in intraperoxisomal SCP$_2$ in Leydig cells *(119)*. Long-term chronic LH treatment also leads to increased SCP$_2$ expression along with hyperplasia, hypertrophy, and increased testosterone secreting capacity in Leydig cells *(120)*. In contrast, LH deprivation is accompanied by a significant reduction in SCP$_2$ levels *(121,122)*. These various results are consistent with the idea that Leydig cell SCP$_2$ plays a significant role in testosterone production.

Factors Directing Cholesterol Movement From OMM to IMMs

The second crucial step in the delivery of cholesterol for steroid hormone production is movement of cholesterol substrate from the OMM to the IMM P450scc site. This step is considered rate-limiting because hydrophobic cholesterol cannot rapidly diffuse through the aqueous intermembrane space of the mitochondria to support acute steroid synthesis, and, as such, requires the participation of cholesterol transfer protein(s) *(8,9,11,91)*. At present, at least two proteins, StAR *(91,123–126)* and the peripheral-type benzodiazepine receptor *(127–130)* are believed to be involved in the translocation of cholesterol from the outer to the IMM (for details *see* Chapter 9).

CONCLUDING REMARKS

In a period of approx five decades, the understanding of the acquisition of cholesterol for Leydig cell steroidogenesis has not progressed as rapidly as it has for the steroidogenic cells of the adrenal gland and ovary. One can only imagine that the reason information on Leydig cell cholesterol uptake and transport has lagged behind is that Leydig cells represent only a minor fraction of total testicular mass, and this low number of cells may have limited the use of many in vivo physiological techniques. In general, cholesterol is a particularly difficult lipid to study because of its hydrophobic nature, its lack of a functional group that can be functionally tagged, and because of its high rate of spontaneous exchange and transfer. Morphological information regarding cholesterol is difficult to obtain because of cholesterol extraction during fixation and processing techniques.

However, following the discovery of SR-BI and its recognition as a bona fide receptor for the uptake of HDL-cholesterol, cholesterol requirements of the Leydig cell have begun to be revisited and compared with other steroidogenic cells. In the rodent, the recent discovery of SR-BI as a promoter of microvillar channel formation, the identity of SR-BI interacting proteins, and the availability of designer Leydig cell lines, SR-BI gene-ablated mice and cholesterol tracers (e.g., BODIPY-CE) have opened up new avenues for cellular and molecular approaches that should advance the understanding of cholesterol trafficking and steroidogenesis in Leydig cells of a variety of animal models.

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9 The Role of StAR in Leydig Cell Steroidogenesis

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SUMMARY

The steroidogenic acute regulatory (StAR) protein mediates the rate-limiting step in steroidogenesis, the transfer of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane, where it is cleaved to form pregnenlone. Its indispensable role in steroidogenesis was demonstrated by showing that mutations in the StAR gene in humans cause the lipoid form of congenital adrenal hyperplasia, a potentially lethal disease resulting from an inability to synthesize steroids. Also, StAR-null mice have a phenotype that is identical to that seen in human mutations. The mechanism whereby StAR mediates the transfer of cholesterol to the inner mitochondrial membrane remains a mystery. This review will attempt to summarize what is currently known about the mechanism of action of StAR and argue that an understanding of the role played by StAR and other proteins in intramitochondrial cholesterol transfer constitutes the biggest challenge in understanding the acute regulation of steroidogenesis.

Key Words: Cholesterol transfer; mitochondria; START domain; steroidogenesis; steroidogenic acute regulatory protein.

INTRODUCTION

The steroidogenic acute regulatory (StAR) protein was initially discovered and characterized over a decade ago. Since that time, numerous observations have been made indicating that this protein plays an indispensable role in the transfer of cholesterol, the substrate for all steroids, from the outer mitochondrial membrane to the inner mitochondrial membrane, the rate-limiting step in steroidogenesis. It was clear that this process required a protein mediator as it was sensitive to protein synthesis inhibitors and in the absence of new proteins, cholesterol does not access the cytochrome-P450 side chain cleavage enzyme in amounts sufficient to support the observed hormonally induced steroidogenesis. Several candidate proteins have been proposed for this role, and their candidacies have been discussed in a number of previous reviews *(1–12)*. Given the limited nature of this review, it will focus mainly on what has been found and what remains to be determined concerning the role of StAR in the mechanism involved in transferring cholesterol to the inner mitochondrial membrane. This remains the single biggest challenge in the quest to understand the acute regulation of steroid biosynthesis. A number of previous review articles have also addressed similar topics *(1,13–20)*.

BACKGROUND

StAR was initially described by Orme-Johnson and colleagues as a 30 kDa phosphoprotein in ACTH-treated rat and mouse adrenocortical cells, in LH-treated rat corpus luteum cells and mouse Leydig cells, and later by Stocco and colleagues in hormone stimulated MA-10 mouse Leydig tumor cells (reviewed in ref. *1*). These proteins were localized to the mitochondria and consisted of several isoforms of a newly synthesized 30 kDa protein. In addition, 37 kDa precursor forms for these proteins containing N-terminal mitochondrial targeting sequences were also detected *(21,22)*. The cDNA for the 37 kDa mitochondrial protein was cloned from MA-10 cells and when compared with other nucleic acid and protein sequences in the database the results indicated this was a novel protein *(23)*. Transfection experiments with StAR cDNA resulted in significant increases in the conversion of cholesterol to pregnenolone *(1,23–25)*, indicating a direct cause and effect role for the 37 and 30 kDa proteins in hormone-regulated steroid production.

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However, the most compelling evidence for the essential requirement for StAR in steroidogenesis came with the finding that mutations in the StAR gene caused the potentially lethal condition known as congenital lipoid adrenal hyperplasia (lipoid CAH) *(25)*. Afflicted individuals are unable to synthesize adequate levels of steroids, are characterized by excessive levels of cholesterol and cholesterol esters in adrenal and testicular steroidogenic cells and do not survive unless steroid replacement therapy is administered. The role of StAR in regulated steroid synthesis was further corroborated when StAR-specific knockout mice were generated and displayed a phenotype essentially identical to the human condition *(26)*. Thus, biochemical and genetic studies demonstrated an indispensable role for StAR in steroid hormone biosynthesis in adrenal and gonadal tissues.

MECHANISM OF StAR ACTION

StAR is required to mediate cholesterol transfer to the inner mitochondrial membrane, but how it performs this function remains an intriguing question. In early studies, the observation that StAR is rapidly imported and processed by the mitochondria resulted in a model hypothesizing that contact sites were formed between the outer and inner mitochondrial membranes during the course of import and that cholesterol could be transferred to the inner membrane through these contact sites *(1,23)*. This was reasonable as the existence of contact sites at loci where proteins were imported into the mitochondria had been described *(27,28)* and, in fact, this possibility continues to be discussed *(29)*. However, it is unlikely that this model is correct because it was later demonstrated that cells expressing StAR proteins lacking the N-terminal 62 amino acids (and thus all of the targeting sequence), supported steroid synthesis to the same extent as wild-type StAR without entering the mitochondria *(30)*. Similarly, recombinant N-62 StAR could fully support steroid synthesis in isolated mitochondria, once again, without entering the organelle *(31)*. In contrast, experiments using constructs truncated in their C-terminus indicated that the cholesterol transferring capability of the StAR protein resided in the C-terminal portion of the molecule *(25,30,32,33)*. This observation is in accord with the finding that most mutations causing lipoid CAH are found in the C-terminal portion of the StAR protein *(7)*, indicating a critical role for this region in cholesterol transfer. The role of the C-terminal region of StAR gained more interest when it was found that a protein known as MLN64 contained an amino acid sequence that was highly homologous to the C-terminus of StAR *(34)*, and significantly stimulated steroid synthesis when transfected into COS-1 cells *(35)*. MNL64 is found in many tissues including the human placenta (which notably lacks StAR), where it was speculated to play a role in steroidogenesis *(13)*. In support of this hypothesis, recombinant MLN64 was recently demonstrated to transfer cholesterol to the inner membrane of isolated placenta mitochondria and increase steroid synthesis sixfold *(36)*. However, MLN64 was localized to the late endosomal compartment, an observation questioning an authentic role for this protein in intramitochondrial cholesterol transfer *(37)*. A possible function for MLN64 in mobilizing cholesterol from the lysosome to the mitochondria in support of steroid biosynthesis was suggested in one study *(38)*, but this theory was severely compromised by the observation that MLN64-null mice had only modest alterations in cellular steroid metabolism *(39)*. Thus, the intracellular function of this StAR homolog protein remains unknown.

An obvious possibility in the search for the mechanism of cholesterol transfer was that StAR interacted with other mitochondrial outer membrane proteins and/or phospholipids to deliver cholesterol to the inner membrane. However, attempts to identify such binding partners using the yeast two-hybrid system, coimmunoprecipitation and binding assays with radioactive StAR and isolated mitochondria have produced only limited results *(14)*. One exception to this was the characterization of a StAR-binding protein (SBP) that was isolated using a yeast two-hybrid system *(40)*. Cotransfection experiments demonstrated that SBP was able to augment steroid production in COS-1 cells, but the specific role of this interesting protein has yet to be determined. Another protein that may interact with StAR in cholesterol transfer is the peripheral benzodiazepine receptor (PBR), a protein found in high levels in the outer mitochondrial membrane of steroidogenic cells *(41–43)*. Using fluorescence energy transfer it was demonstrated that StAR and PBR are closely associated on the outer mitochondrial membrane, approx 100 Å from each other *(44)*. Based on this association, the authors proposed a model in which StAR targets cholesterol to the PBR that then facilitates its transfer to the inner mitochondrial membrane. In support of a StAR/PBR interaction, subsequent studies have shown StAR and PBR function in a coordinated manner to supply cholesterol for steroid biosynthesis *(45,46)*. Although PBR is involved in cholesterol transfer to the inner mitochondrial membrane, little is known concerning the exact mechanism of its action in this process or the nature of its interaction with StAR. This is further complicated by the observation that StAR

promoted cholesterol transfer into mitochondria in which outer membrane proteins were removed by partial proteolysis *(47)*, and in artificial vesicles devoid of other proteins *(48)* suggesting that StAR does not have protein binding partners on the outer mitochondrial membrane. The relationship between StAR and PBR constitutes an extremely interesting area that will require further study.

In one early effort to explain StAR action, Kallen et al. *(47)*, demonstrated that StAR acted as a sterol transfer protein that enhanced the desorption of cholesterol from one sterol containing membrane to another through alterations in the membrane produced by the C-terminal region. The transfer of cholesterol was specific in that identical experiments employing phosphatidylcholine failed to result in transfer of this phospholipid. That StAR can function in this manner in vivo remains to be proven. A different approach by Miller and colleagues studied the physical characteristics of the StAR protein under different localized conditions and utilized these characteristics to provide insights into the mechanism of StAR action. StAR was subjected to limited proteolysis at different pH values and behaved differently as the pH decreased *(49)*. At pH 3.5–4.0, StAR underwent partial unfolding and a transition to a molten globule state, a structure within proteins characterized by a loss of tertiary structure but a retention of virtually all of the secondary structure. They argued that the pH microenvironment of the mitochondria could cause formation of the molten globule state because of the expulsion of protons from the matrix and/or the presence of negatively charged head groups on the phospholipids. As the transition to a molten globule occurs, the resulting structural change could produce an opening of the StAR protein possibly exposing a hydrophobic region or, it may prolong the interval with which StAR can reside on the outer membrane, thus allowing increased transfer of cholesterol during this period.

START DOMAINS

The cholesterol-transferring region of the StAR protein is located in the C-terminal region of the protein as demonstrated with both N-terminally truncated StAR proteins and MLN64. Ponting and Aravind *(50)*, demonstrated that sequences in the C-terminus of StAR are homologous to sequences in several other proteins, including MNL64, which display diverse functions. They named these sequences START domains, for StAR related lipid transfer domains. START domains consist of 200–210 amino acid stretches capable of binding lipids, and the possibility that StAR was a lipid binding carrier protein would have to be considered. This possibility received an exciting boost when Tsujishita and Hurley *(51)*, succeeded in obtaining crystals and solving the structure for the START domain of the MNL64 protein. They demonstrated that both StAR-START and MLN64-START could bind cholesterol in an identical manner at a ratio of 1:1. The crystal structure of MLN64-START illustrated that it consisted of an $\alpha + \beta$-fold built around a U-shaped incomplete β-barrel and contained a nine-stranded antiparallel β-sheet, 4-α-helices, and 2-Ω-loops. Most importantly, the tertiary structure of MLN64-START revealed a hydrophobic tunnel that was of sufficient size to bind a single molecule of cholesterol. Based on these findings the authors proposed that StAR functioned in transferring cholesterol to the inner mitochondrial membrane through its ability to act as an inter-mitochondrial membrane cholesterol shuttling protein. This model is in direct conflict with the finding that StAR acts solely on the outer mitochondrial membrane.

Whereas the controversy of whether StAR can act as a cholesterol shuttling protein in the intermembrane space or whether it can act on the outer mitochondrial membrane to effect cholesterol transfer continues, the Miller laboratory has continued to provide new structure–function studies. In one study they examined the structural properties of a recombinant 63–193 protease-resistant StAR protein *(52)*. Expression of the 63–193 domain in the absence of the 194–285 molten globule domain altered its structure rendering it more susceptible to protease digestion and devoid of tertiary structure. Importantly, addition of 63–193 StAR to phosphatidylcholine or phosphatidylserine containing liposomes induced the formation of stable protein–liposome complexes, indicating that the N-terminal region of the StAR protein can form a molten globule and that this structure can interact directly with membranes. This finding is important because when in the molten globule state, proteins lose tertiary structure and can become partially opened. This process could expose a potential hydrophobic interior that would allow them to interact with phospholipid membranes. This observation has important implications in that StAR has been shown to closely interact with the outer mitochondrial membrane during the course of cholesterol transfer, and this interaction apparently does not require it to bind with other proteins *(47)*.

Studies on the interactions of StAR with artificial membranes continued. Utilizing unilamellar artificial membranes made up of phosphatidylcholine, or phosphatidylcholine:cholesterol *(53)*, it was demonstrated

that recombinant StAR readily bound to these membranes in the absence of other proteins, supporting the hypothesis that StAR can interact directly with the outer mitochondrial membrane. In addition, this binding occurred maximally at low pH values, conditions favoring the formation of molten globule structures. The degree of binding of StAR to these membranes varied with the heterogeneity of the membrane composition and StAR was able to bind preferentially to the cholesterol rich domains in cholesterol containing membranes. It is intriguing to speculate that StAR binds to such regions in the relatively cholesterol rich mitochondrial outer membrane to more easily facilitate transfer of this substrate to the cholesterol poor inner membrane, but information on this possibility is lacking. Notably, StAR proteins with mutations that cause lipoid CAH did not bind to the artificial membranes as efficiently as did wild-type StAR *(54)*. These studies also showed that when StAR bound to artificial membranes containing cardiolipin in concentrations approximating that found in mitochondrial outer membranes, it underwent a conformational change to a molten globule more readily than when cardiolipin-free membranes were used. Interestingly, it has recently been shown that at a pH low enough to cause molten globule formation, StAR can bind to small unilamellar vesicles constructed to resemble the mitochondrial outer membrane through an interaction with the C-terminal α-4 helix *(55)*. Another observation demonstrating the ability of StAR to transfer cholesterol in the absence of other proteins was made in artificially constructed donor and acceptor phospholipid vesicles containing cholesterol, and P450scc, respectively. Addition of recombinant StAR to these vesicles increased P450scc activity 5–10-fold as a result of the specific transfer of cholesterol from one vesicle to the other *(48)*.

Studies using a fluorescent cholesterol analog demonstrated that the binding of cholesterol to StAR induced a change in its secondary structure and also showed that StAR could enhance sterol transfer into mitochondria by 100-fold *(56)*. This study demonstrated the specificity of StAR action in that sterol transfer in steroidogenic mitochondria was 67-fold higher than sterol transfer in nonsteroidogenic mitochondria. That StAR appeared to act solely on the outer mitochondrial membrane was further shown using a series of cDNA constructs consisting of StAR attached to the leader sequences of other mitochondrial proteins *(57)*. The results of these studies demonstrated that StAR acts on the outer mitochondrial membrane and if its residence time is longer on the outer membrane, more amount of steroid was synthesized, indicating that cholesterol transfer occurs during the period of time StAR is associated with the outer membrane. In contradiction, another study showed that it was the targeting of the phosphorylated 30 kDa mature form of StAR to the inner mitochondrial membrane, which was responsible for the majority of cholesterol transfer and hence, steroid production *(58)*. The situation is further complicated by observations obtained using molecular modeling and structure-based thermodynamics which concluded that the results were consistent with StAR functioning either in the mitochondrial intermembrane space or on the outer membrane *(59)*. The discrepancy between these observations has not yet been resolved.

StAR HOMOLOGS

To add more interest to this field, additional StAR homologs have been recently discovered and are currently being characterized. The basis of their homologies is the presence of a START domain in each protein. To date, 15 mammalian proteins designated as STARD1 through STARD15 which can be grouped into six subfamilies have been found. One of the first StAR homolog proteins identified was StarD4, a protein expressed in response to a high cholesterol diet in many tissues with the highest levels found in liver and kidney *(60)*. StarD5 is also expressed in high levels in liver and kidney and StarD6 is expressed only in the testis, but neither of these genes is sterol regulated. The crystal structure of StarD4 indicates that it is similar to MLN64 and, by inference, to the START domain of other StAR related proteins *(61)*. Although unknown, the function of these StAR homologs appears to be associated with lipid binding and transfer activities *(62,63)*. Recently, reviews of the START domain family of proteins have been published *(64–66)*, but it is clear that the studies on the function of these homologs are still in their infancy.

CONCLUSIONS

In the ten plus years since the StAR protein has been discovered, a large volume of work has been performed in an effort to determine its function, structure, the signaling pathways involved in its expression, its transcriptional regulation, and its mechanism of action. Great strides have been made in each of these categories. We know the function of StAR is to mediate the transfer of cholesterol from the outer to the inner mitochondrial membrane. Whether StAR accomplishes this alone or in conjunction with other proteins is unknown, but PBR, SBP, and perhaps other unknown proteins may be intricately involved in this process. Although the crystal structure of StAR (STARD1) has not yet been solved,

the structures of the START domains of the closely related MLN64 and STARD4 proteins have been determined. The highlight of each structure is the presence of a hydrophobic pocket that can bind one molecule of cholesterol per molecule of protein and it is clear from previous studies that a functional START domain that can bind cholesterol is instrumental in its action. The signaling pathways involved in induction of StAR expression are complex and once again excellent progress is being made in this area (reviewed in ref. *67*). The regulation of the transcriptional expression of StAR has also been widely studied. The StAR promoter has been shown to be very complex and is regulated by a variety of transcription factors, cofactors, repressors, and corepressors that vary from species to species, and tissue to tissue.

The area that has proven to be most refractory to solution is that of StAR's role in the mechanism involved in transferring cholesterol across the mitochondrial membranes. It is within this realm therefore, that the greatest challenges concerning StAR are to be found. The two models that have the most credence are the intermembrane shuttle model and the molten globule model. In the former StAR acts as a carrier of cholesterol from the outer to the inner mitochondrial membrane and in the latter StAR acts to promote cholesterol transfer through changes in its conformation that might produce a hydrophobic tunnel or region through which cholesterol might pass. Each model has strong points and each model has facets that appear to be incompatible with the other. For example, the cholesterol shuttle model is inconsistent with the observation that StAR can act on the outer mitochondrial membrane and promote cholesterol transfer without ever entering the intermembrane space or matrix. As for the molten globule hypothesis, the data clearly indicates that StAR can form this structure at low pH, but does it ever do so in vivo? Also, as PBR and SBP have been shown to interact with StAR and enhance steroid synthesis, the exact mechanisms involved in these interactions are going to be crucial in understanding the cholesterol transfer process. At this juncture in time it appears that we will only know the answers to these questions after a considerable amount of additional work. As such, the mechanism of action of StAR in mediating cholesterol transfer in steroidogenic mitochondria remains, perhaps, the most intriguing question to be answered in the field of steroidogenesis.

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10 Steroidogenic Enzymes in Leydig Cells

Anita H. Payne, PhD

SUMMARY

This chapter describes the enzymes expressed in Leydig cells that are required for the biosynthesis of testosterone from cholesterol, as well as the two enzymes, steroid 5α-reductase and P450arom, that metabolize testosterone to dihydrotestosterone and estradiol, respectively. The emphasis is on human and mouse enzymes.

Key Words: CYP11A; CYP17A; CYP19A, 3β-HSD; 17β-HSD; Leydig cell; P450arom; P450c17; P450scc; steroidogenesis; steroid-α-reductase.

INTRODUCTION

The Leydig cell is the only cell in the male that expresses all of the enzymes essential for the conversion of cholesterol to testosterone, the major male sex hormone secreted by the testis. Testosterone can be metabolized further in the Leydig cell by S5A (5α-reductase) to yield dihydrotestosterone *(1)*, or by the enzyme, CYP19A1 (P450arom) to yield estradiol *(2,3)*. Testosterone or its metabolite, dihydrotestosterone, is essential for male sexual differentiation, expression of male secondary sex characteristics *(4)*, and maintenance of spermatogenesis *(5)*. The biosynthesis of testosterone is dependent on both acute and chronic stimulation of Leydig cells by the pituitary hormone luteinizing hormone (LH). LH binds to specific high affinity receptors on the surface of Leydig cells activating adenylate cyclase, resulting in increased production of cyclic AMP. The acute stimulation results in the rapid transport of cholesterol from the outer to the inner mitochondrial membrane, the site of the first enzyme in the pathway of cholesterol to testosterone. This process is mediated by the steroidogenic acute regulatory protein (StAR) *(6), see* Chapter 9. Chronic stimulation of Leydig cells by LH or cAMP is required for optimal expression of the enzymes required for the biosynthesis of testosterone from cholesterol. This chapter describes the enzymes involved in the biosynthesis of testosterone from cholesterol as well as the two enzymes, steroid 5α-reductase and P450arom that metabolize testosterone to dihydrotestosterone and estradiol, respectively (Fig. 1). The enzymes can be divided into two major classes of proteins: the cytochrome P450 heme-containing proteins CYP11A1 (P450scc), CYP17A1 (P450c17), and P450arom, and the hydroxysteroid dehydrogenases 3β-hydroxysteroid dehydrogenase (3β-HSD), and 17β-hydroxysteroid dehydrogenase (17β-HSD). 5α-reductase belongs to the steroid 5α-reductase family (Table 1C; ref. *7*).

The initial step in the biosynthesis of testosterone from cholesterol is the conversion of the C27 cholesterol to the C21 steroid, pregnenolone. This reaction is catalyzed by the cytochrome P450 enzyme, cholesterol side-chain cleavage (P450scc), located in the inner mitochondrial membrane. Pregnenolone diffuses across the mitochondrial membrane and is further metabolized by enzymes associated with the smooth endoplasmic reticulum. These include the cytochrome P450 17α-hydroxylase C17-C20 lyase, P450c17, which catalyzes the conversion of the C21 steroids pregnenolone or progesterone to the C19 steroids dehydroepiandrosterone or androstenedione, respectively, and 3β-hydroxysteroid dehydrogenase which catalyzes the conversion of the Δ5 steroids, pregnenolone, or dehydroepiandrosterone to the Δ4 steroids, progesterone, or androstenedione, respectively, and 17β-hydroxysteroid dehydrogenase, which catalyzes the final step in the biosynthesis of testosterone (Fig. 1).

CYTOCHROME P450s

The cytochrome P450 enzymes are members of a superfamily of heme-containing proteins *(8)*. They

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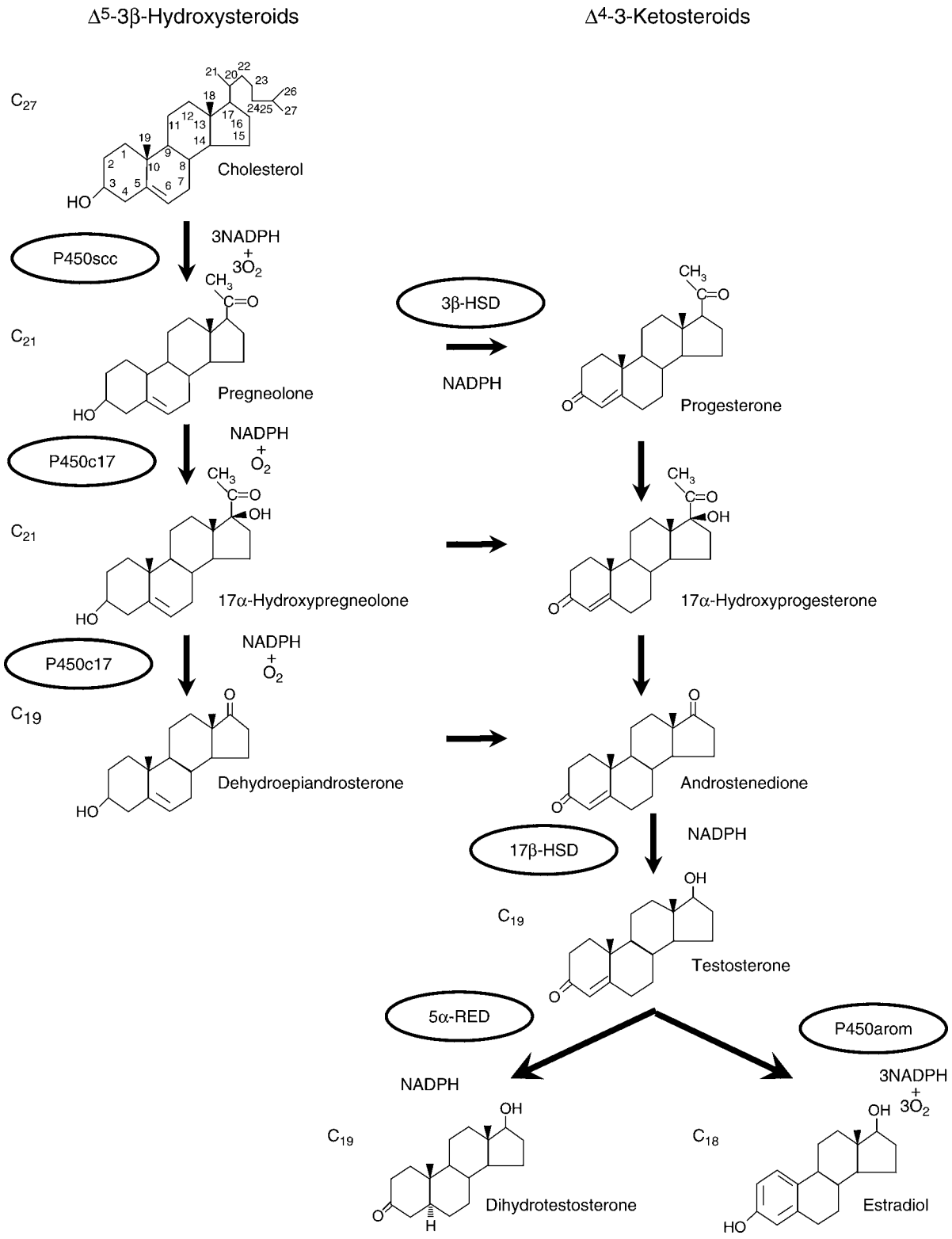


Fig. 1. Steroid biosynthetic pathways in Leydig cells.

derive their name from the characteristic which, when complexed in vitro with exogenous carbon monooxide, absorb light maximally at 450 nm. They function as monooxygenases utilizing reduced nicotinamide adenine dinucleotide phosphate (NADPH) as the donor for the reduction of molecular oxygen. The general reaction is: $RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$. In this reaction, the oxygen is activated by P450, and one oxygen atom is introduced into the substrate RH as a hydroxyl group, and the other oxygen is reduced to H_2O. The electrons from NADPH are transferred to the substrate by two distinct electron transfer systems. The mitochondrial transfer involves the transfer of high potential electrons to a flavoprotein, adrenodoxin reductase (ferredoxin reductase), and then sequentially to adrenodoxin (ferredoxin), a nonheme iron–sulfur protein, then P450, and finally, to the substrate. The microsomal electron transfer system involves only one protein, cytochrome P450 oxidoreductase, a protein that contains two flavins. The electrons are transferred from NADPH, to flavinadenine dinucleotide, followed sequentially by transfer to flavinmononucleotide and then the substrate *(7)*.

P450scc

Reaction Catalyzed

P450scc catalyzes the conversion of cholesterol to pregnenolone, the first and rate-limiting enzymatic step in the biosynthesis of testosterone (Fig. 1). P450scc catalyzes three sequential oxidation reactions of cholesterol. Each reaction requires one molecule of oxygen and one molecule of NADPH, and the mitochondrial electron transfer system *(7)*. The first reaction is the hydroxylation at C22, followed by hydroxylation at C20 to yield 20,22R-hydroxycholesterol that is cleaved between C22 and C20 to yield the C21 steroid pregnenolone, and isocapraldehyde *(9,10)* Isocapraldehyde is further metabolized to isocaproic acid *(11)*.

Investigations utilizing the purified protein as well as studies on recombinant proteins from P450scc cDNAs have provided conclusive evidence that a single protein catalyzes all three reactions at a single active site *(12,13)*. The pair of electrons required for each of the reactions is transferred from NADPH to a flavoprotein, ferredoxin reductase, and then sequentially to a nonheme iron–sulfur protein, ferredoxin, to P450scc, and finally, to the substrate *(14)*. The P450scc enzyme is typical of all mitochondrial cytochrome P450 enzymes that share the same electron transfer proteins *(15)*. It has been shown that the P450scc enzyme only functions in the mitochondrion. This requirement appears to be for the mitochondrial environment rather than the specific mitochondrial electron transfer system *(16)*. A model of the interactions between P450scc and the electron transport proteins has been proposed based on the expression of mutants. The results of these studies indicate that the acidic residues, Asp 76, and Asp 79, of ferredoxin interact with the basic residues of ferredoxin reductase and P450scc *(17)*.

Molecular Structure

P450scc is the product of a single gene. The cDNA was first isolated in 1984 from bovine adrenal cortex mRNA *(18)*. Subsequently, P450scc cDNA has been cloned from human *(19)*, rat *(20)*, mouse *(21)*, and numerous other species. The deduced amino acid (aa) sequence displays high homology among species, equal to or more than 71%. The open reading frame of human cDNA encodes a peptide consisting of 521 aa *(19,22)*. The 39 aa at the amino-terminus includes the N-terminal leader sequence essential for the translocation of the protein to the inner mitochondrial membrane. The removal of this leader sequence yields a protein of 482 aa *(16,19)*. The aa sequence contains a heme-binding region common to the P450 superfamily located close to the carboxyl terminus containing a single cysteine residue *(18)*, and a specific 20 aa region of high homology among species located at the amino-terminus which is proposed to be the P450scc-specific substrate binding region *(23)*.

The structure of the cholesterol-side chain cleavage gene designated as *CYP11A1* has been determined in human *(22)* and rat *(24)*. The gene is at least 20 kb in length, and consists of nine exons containing an unusual exon/intron junctional sequence that begins with GC found in the sixth intron of both the human gene *(22)* and the rat gene *(24)*. The human gene is located on chromosome 15q23-q24 *(19)*, and the mouse gene is found on chromosome 9 at 31 cM *(25)*.

P450c17

Reaction Catalyzed

P450c17 catalyzes two mixed function oxidase reactions, 17α-hydroxylation, and C17–C20 cleavage. Each reaction requires one molecule of NADPH, and one molecule of oxygen, and the microsomal electron transfer protein, cytochrome P450 oxidoreductase *(7)*. The two reactions catalyzed by P450c17 are the 17α-hydroxylation of either the Δ5-C21 steroid, pregnenolone, or the Δ4-C21 steroid, progesterone, followed by cleavage of the C17-20 bond to yield either the C19 steroids, dehydroepiandrosterone (DHEA), or androstenedione, respectively (Fig. 1). In this two

Table 1
Leyding Cell Steroidogenic Enzymes

Gene		*Chromosomal location*		*Protein name (molecular mass)*	*Subcellular location*	*Synonyms*	*Protein Gene ID (accession no)*	
Human	*Mouse*	*Human*	*Mouse*				*Human*	*Mouse*
A. Cytochrome P450								
CYP11A1	*Cyp11a1*	15q23-q24	9 (31 cM)	CYP11A1 (56 kDa)	Mitochondria *(146)*	P450scc, cholesterol side chain cleavage, cholesterol desmolase, cytochrome P450 cholesterol side chain cleavage, cholesterol 20-22 desmolase	1583 (P05108)	13070 (Q9QZ82)
CYP17A1	*Cyp17a1*	10q24.3	19 (46 cM)	CYP17A1 (57 kDA)	Microsomal *(146)*	P450c17, 17α- hydroxylase/ 17,20 lyase, P450 17α-hydroxylase/ c17-20 lyase	281739 (P05093)	13074 (P27786)
CYP19A1	*Cyp19a1*	15q21.1	9 (31 cM)	CYP19A1 (58 kDa)	Microsomal *(57)*	Aromatase, P450arom, cytochrome P450arom, estrogen syntase	1588 (P11511)	13075 (P28649)
B. Hydroxysteroid dehydrogenases								
HSD3B1	*Hsd3b6*	1p13.1	3 (F2.2)	3β-HSDI human 3β-HSD VI mouse (42 kDa)	Microsomal	3β-HSD type-I (type-VI), 3β-HSD/Δ 4–Δ5 isomerase, 3β-hydroxyΔ5-steroid dehydrogenase, 3β-hydroxy-5-ene steroid dehydrogenase	3283 (P14060)	15497 (O35469)
HSD3B2	*Hsd3b1*	1p13.1	3 (49.8 cM)	3β-HSDII human 3β-HSDI mouse (42 kDa)	Mitochondria and microsomal *(146)*	*See* previous entry	3284 (P26439)	15492 (P24815)
HSD17B3	Hsd17b3	9q22	13	17β-HSD3 (34.5 kDa)	Microsomal (147)	17β-HSD 3, testicular 17β-HSD	3293 (P37058)	15487 (P70385)
C. Steroid 5α-reductases								

SRDA1	*Srda1*	5p15	13 (39 cM)	SRDA1 (29.5 kDa) human (24.9 kDa) mouse	Microsomal *(148)*	3-oxo-5α-reductase, 5α-reductase	6715 (P18405)	24950
SRDA 2	*Srda1*	2p23	17 (E2)	SRDA2 (28.4 kDa) human (24.9 kDa) mouse	Microsomal *(148)*	*See* previous entry	6716 (P31213)	94224 (Q99N99)

Swiss-Prot http://us.espasy.org/cgi-bin/sprot-search.ac.

step reaction, 17α-hydroxypregnenolone or 17α-hydroxyprogesterone is synthesized as an intermediate. Initially, it was believed that each reaction was catalyzed by a distinct enzyme, but studies by Hall and colleagues *(26,27)* demonstrated that a single purified protein catalyzed both 17α-hydroxylation and C17–C20 cleavage (lyase) activity. Subsequent cloning of a bovine P450c17 cDNA and expression of this cDNA confirmed that both reactions were catalyzed by the same protein *(28)*. Although, the P450c17 enzyme from various species catalyzes both the hydroxylation and the lyase reaction, there are marked species-dependent differences in the utilization of either 17α-hydroxypregnenolone (Δ5) or 17α-hydroxyprogesterone (Δ4) as substrate for the lyase reaction. The human and bovine enzymes use 17α-hydroxypregnenolone as the preferential substrate yielding DHEA as the product, whereas the rodent enzyme utilizes 17α-hydroxyprogesterone as the substrate yielding androstenedione as the product *(29)*. Auchus and colleagues provided conclusive evidence that human testes synthesize testosterone predominantly by the Δ5 pathway *(30)*. These species-dependent differences in substrate preference for the C17–C20 lyase activity are not related to differences in the aa sequence of the bovine and human enzyme, compared with the rodent enzyme. However, the differences are a property of the human and bovine enzyme required for high molar concentration of cytochrome P450 reductase *(31,32)*, serine/threonine phosphorylation of P450c17 *(33,34)*, and the accessory protein cytochrome b_5 in promoting lyase activity of 17α-hydroxypregnenolone, but not of 17α-hydroxyprogesterone *(29,35)*.

Additional activities to that of the classic 17α-hydroxylation/C17–C20 cleavage have been observed. Swart et al. *(36)* reported that human P450c17 also expresses 16α-hydroxylase activity at the same site as 17α-hydroxylase activity. More recent study by Liu et al. *(37)* using a P450c17 deficient MA10 mouse tumor Leydig cell culture indicated that P450c17 expresses a secondary activity, squalene monooxygenase (epoxidase) activity, suggesting that this enzyme may also be involved in cholesterol biosynthesis as proposed several years earlier by Lieberman and Warne *(38)*.

Molecular Structure

Genomic Southern blotting and/or cloning has established that in mouse *(39)*, rat *(40)*, and human *(41,42)* as well as in other species there is a single gene designated as *CYP17A1* in human and *Cyp17a1* in mouse. The *CYP17A1* gene is approx 6 kb in length and contains eight exons with the location of intron–exon boundaries conserved among species. The 5′ upstream region of the human, bovine, porcine, rat, and mouse gene share a high homology over the first 550 bp including the same nonconsensus TATA box *(41–46)*. The human *CYP17A1* gene has been mapped to chromosome 10q24.3 *(47,48)* and the mouse *Cyp17a1* gene to chromosome 19 at 46 cM *(39)*. The human P450c17 protein contains 508 aa *(49)* compared with 507 aa in the mouse *(39)* and rat *(40)* proteins. The molecular mass of the P450c17 protein is approx 57 kDa. Comparison of the mouse aa sequence to rat and human sequences indicates that they are 83 and 66% identical, respectively. The P450c17 protein of different species contains regions of high homology common to members of the P450 gene family *(39)*. These are the putative binding regions for mouse aa 434–454 *(39)*, human aa 435–455 *(50)*, and the ozols tridecapeptide sequence (343–372 aa) *(51)* that may play a role in substrate specificity *(52)*. In addition, there is a region that is specifically conserved among different species of P450c17 (296–319) that may function in catalysis *(53)*. Arginine346 in the rat enzyme *(54,55)* and arginine347 in the human enzyme were found to be critical for catalyzing lyase activity.

P450arom

Reaction Catalyzed

P450arom catalyzes the conversion of the C19 androgens, androstenedione, and testosterone, to the C18 estrogens, estrone, and estradiol, respectively. The reaction requires three molecules of oxygen and three molecules of NADPH using the microsomal electron transfer system. The first two oxygen molecules are required for the oxidation of the C19 methyl group by standard hydroxylation reactions, whereas the third oxygen molecule is required for a reaction proposed to be a peroxidative attack on the C19 methyl group combined with elimination of the 1β hydrogen to yield a phenolic A ring and formic acid *(7)*.

Molecular Structure

P450arom (*CYP19A1*) is the product of a single gene in human *(56,57)*, mouse *(58)*, and rat *(59)*. The human gene has been mapped to 15q21.1 *(60)*, and the mouse gene is located on chromosome 9 at 31 cM *(25)*. The human gene contains 10 exons, nine of which includes the coding region spanning approx 30 kb *(56)*. Upstream of exon II are several alternative exon 1s that are spliced into the 5′ untranslated region, which determines the tissue-specific expression of the protein *(56)*. The proximal promoter II determines testicular and ovarian expression of P450arom, and the transcript originates immediately upstream of the

translational start site, approx 26 bp downstream of the putative TATA sequence *(57,61,62)*. Although, the transcripts have different termini in the different tissues, the coding region of the expressed protein is identical.

The deduced aa sequence of human P450arom in comparison to the rat and mouse protein exhibits 81% homology *(56)*. Both the human and mouse protein consist of 503 aa with a molecular mass of 58 kDa *(56)*. The P450arom proteins from different species contain the same structural features described for the other cytochrome P450 enzymes: the heme-binding region containing a conserved cysteine residue that serves as the fifth coordinating ligand of the heme iron, and the substrate binding site in the amino-terminal I-helix region.

HYDROXYSTEROID DEHYDROGENASES

The hydroxysteroid dehydrogenases, 3β-HSD and 17β-HSD, involved in the biosynthesis of testosterone from cholesterol, belong to the same phylogenetic protein family, namely the short-chain alcohol dehydrogenase reductase superfamily. In general, they are involved in the reduction and oxidation of steroid hormones, requiring $NAD^+/NADP^+$ as acceptors and their reduced forms as donors of reducing equivalents. One of the major differences between the P450 enzymes and the hydroxysteroid dehydrogenases is that each of the P450 enzymes is a product of a single gene, whereas there are several isoforms for 3β-HSDs and several isozymes of the 17β-HSDs, each a product of a distinct gene. The number of isoforms or isozymes varies in different species, in tissue distribution, catalytic activity (whether they function predominantly as dehydrogenases or reductases), substrate and cofactor specificity, and subcellular distribution. The 3β-HSD isoform expressed in Leydig cells is 3β-HSD II in human *(63)*, 3β-HSD I *(64)* and VI *(65)* in mouse, and 3β-HSD I in rat *(66)* The 17β-HSD3 is the 17β-HSD isoform exclusively expressed in both human *(67)* and rodent adult Leydig cells *(68,69)*.

3β-Hydroxysteroid Dehydrogenase

Reaction Catalyzed

The 3β-HSD isoforms expressed in Leydig cells catalyze the conversion of the Δ5-3β-hydroxysteroids, pregnenolone, 17α-hydroxypregnenolone, and DHEA, to the Δ4-3-ketosteroids, progesterone, 17α-hydroxyprogesterone, and androstenedione, respectively. Two sequential reactions are involved in the conversion of the Δ5-3β-hydroxysteroid to a Δ4-3-ketosteroid. The first reaction is the dehydrogenation of the 3β-equatorial hydroxysteroid, requiring the coenzyme NAD^+, yielding the Δ5-3-keto intermediate, and reduced NADH. The reduced NADH, activates the isomerization of the Δ5-3-keto steroid to yield the Δ4-3-ketosteroid *(7,70,71)*. This reaction is catalyzed by a single dimeric protein without the release of the intermediate or coenzyme *(71)*. Four isoforms in rat have been identified *(7,72)*. Each of these isoforms is the product of a distinct gene. Human *HSD3B* genes are found on chromosome 1p31.1 *(73)* and the *Hsd3b* mouse genes are located in a cluster on mouse chromosome 3 close to the centromeric region that shows conservation of gene order and physical distance with the centromeric region of human chromosome 1 *(74,75)*. All of the *HSD3B* genes consist of four exons, with the start site of translation found in exon 2 *(75)*. The two human genes are approx 7.8 kb, and their nucleotide sequences are highly homologous including their intronic sequences and the 1250 bp sequence of the 5′ flanking region that exhibits 81.9% identity *(72)*. The size of the mouse genes varies as result of differences in the size of their introns *(75,76)*. Intron 1 of the mouse *Hsd3b6* gene was found to be 3.1 kb *(76)* compared with 126, 125, and 132 bp found in mouse *Hsd3b1* and human *HSD3B1* and *HSD3B2 (75)*. The open reading frames of mouse I and mouse VI 3β-HSD, the isoforms expressed in Leydig cells, encode a protein including the initiator methionine of 373 aa *(65)*, whereas human II 3β-HSD encodes a protein of 372 aa *(72)*. The aa sequences among the different isoforms and between mouse and human isoforms show a high degree of identity. Mouse 3β-HSD I is 84% identical to mouse VI, and 71% identical to human II *(65,72)*. Although the aa sequence predicts a molecular mass of 42 kDa for all of the 3β-HSD proteins, when subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the mobilities of mouse 3β-HSD I and VI are distinct, exhibiting an apparent molecular mass of 42 and 44 kDa, respectively *(65)*. The cofactor binding site is located in the amino-terminal sequence. Investigations using homology modeling of human 3β-HSD I demonstrated that Asp36 is responsible for the NAD(H) binding site *(71)*. In earlier studies investigating the difference in the aa sequence of mouse 3β-HSD I, which requires NAD^+ as a cofactor, and mouse 3β-HSD IV and V, which require $NADP^+$ as cofactor, it was found that Asp36 was essential for NAD^+-mediated dehydrogenation/isomerization, and replacement of Asp36 with phenylalanine at position 36 changed the cofactor specificity to $NADP^+$. *(77,78)* The dehydrogenase activity has been localized to the Y154-P-H-S-K158 domain and the isomerase site to Tyr269 and Lys273 of the human 3β-HSD protein *(79)*.

17-Hydroxysteroid

Till date, 11 distinct 17β-HSDs have been identified. Unlike the 3β-HSDs described earlier, there is little homology among the different 17β-HSD isozymes *(7)*. But unlike the 3β-HSDs, the orthologs among the different species are assigned the same number. Thus, the isozyme expressed in Leydig cells is 17β-HSD 3 in human, rat, and mouse.

Reaction Catalyzed

The 17β-HSD catalyzes the last step in the biosynthesis of testosterone. It converts androstenedione, a weak androgen, to testosterone, a potent androgen. 17β-HSD 3 prefers NADPH as a cofactor, and its primary activity is reductive.

Molecular Structure

The human gene *HSD17B3* maps to chromosome 9q22 *(67)* and the mouse gene *Hsd17b3* is located on chromosome 13 *(68)*. The human gene is 60 kb in length and contains 11 exons *(67)*. It encodes a protein of 310 aa with a molecular mass of 34.5 kDa and no apparent membrane spanning region *(67)*. The mouse gene encodes a protein of 305 aa, five fewer than the human with a molecular mass of 33.7 kDa *(68)*. The mouse protein is missing four of the aa at the amino-terminus and Val245 of the human sequence; the aa identity between the mouse and the human protein is 72.5%, and similarity is 94.8% *(68)*. Site-directed mutational analysis of human 17β-HSD 3 demonstrated that arginine at position 80 was critical for binding of the cofactor NADPH *(80)*.

STEROID 5α-REDUCTASE

Reaction Catalyzed

Steroid 5α-reductase (3-oxo-5α-steroid reductase) catalyzes the irreversible conversion of a 3-keto Δ4–Δ5 structure to the corresponding 5α-reduced metabolite, e.g., testosterone to 5α-dihydrotestosterone (Fig. 1) or progesterone to 5α-dihydroprogesterone. NADPH is the donor of the electrons. Conversion of testosterone to dihydrotestosterone is predominant in androgen target tissues, although, it is also present in the testis. In several species, including rat *(81,82)* and mouse *(83,84)*, there is a peak of 5α-reductase activity in Leydig cells during pubertal development. The major androgen produced in mouse Leydig cells during pubertal development is 5α-androstanediol reaching a maximal production between 25 and 30 d postnatal *(83)*.

Molecular Structure

Two isoforms of steroid 5α-reductase, each a product of a distinct gene, have been identified in human *(85)*, rat *(85)*, and mouse *(86)*. Both the human, and most likely the mouse and rat genes, contain five exons with the positions of the introns being essentially identical in the two isoforms *(85)*. The two genes, human *SDR5A1* and *SDR5A2*, are located on distinct chromosomes. *SDR5A1* maps to the distal arm of chromosome 5p15 *(87)*, whereas *SDR5A2* is located on chromosome 2p23 *(88)*, the mouse *Sdr5a1* maps to chromosome 13 39.0 cM and mouse *Sdr5a2* to 17E2.

The steroid 5α-reductases are hydrophobic intrinsic membrane-bound proteins: the human 5α isozyme type-1 consists of 259 aa whereas the type-2 consists of 254 aa with a molecular mass of 29.5 kDa and 28.4 kDa, respectively (Table 1C; *89*). The respective mouse 5α-reductases 1 and 2 consist of 217 and 254 aa, and a molecular mass of 24.9 kDA and 28.6 kDa, respectively (Table 1C; ref. *90*). The enzymes show aberrant electrophoretic mobilities in sodium dodecyl sulfate polyacrylamide gels. The human isozymes migrate with molecular weights of 21–27 kDa instead of the predicted 28 and 29 kDa *(85)*.

REGULATION OF EXPRESSION OF STEROIDOGENIC ENZYMES IN LEYDIG CELLS

P450 Enzymes

A major nuclear factor that is essential for cell-specific expression for P450 steroidogenic enzymes was identified by two laboratories in 1992. This nuclear DNA-binding protein, referred to as SF-1 by Lala et al. *(91)* or Ad4BP by Morohashi et al. *(92)* belongs to the orphan nuclear receptor family and binds to variants of an AGGTCA sequence motif found in the proximal promoter of all P450 steroidogenic enzymes *(93,94)*. Although SF-1 is essential for cell-specific gonadal expression, other factors are necessary for determining maximal as well as cell-specific expression of these enzymes.

Chronic stimulation of Leydig cells by the pituitary hormone LH is required for the maintenance of optimal expression of the enzymes. LH, acting via G protein-coupled receptors, activates adenylate cyclase thereby, increasing cAMP, which in turn, leads to increased synthesis of P450 steroidogenic enzymes. The regulation of LH stimulation via cAMP is not mediated by the cAMP response element (CRE/CRE-binding protein [CREB]) system with the exception of *CYP19A1*. It has been reported that cAMP acts via CRE/CREB in the rat *Cyp19a1* promoter expressed in rat granulosa cells and in R2C Leydig cells *(95)* and in the PII human *CYP19A1* promoter expressed in human granulosa cells

(96). cAMP-responsive sequences found in the promoters of *CYP11A1* and *CYP17A1* differ among these *CYP* genes and among the same genes of different species *(45,97)*. Although, hormone-stimulated increases in cAMP enhance the expression of all of steroidogenic P450 enzymes, additional factors are involved in maintaining maximal expression. Recent studies have provided evidence for a role of GATA-4 and GATA-6 as phosphorylated intermediates in cAMP-stimulated expression of P450scc, P450c17, P450arom, and 3β-HSD *(98–100)*.

In vitro studies using isolated Leydig cells in culture have contributed to our understanding of the regulation of steroidogenic enzymes. Early studies reported that treatment of immature porcine Leydig cells in culture with hCG increased the *de novo* synthesis of P450scc and adrenodoxin *(101)*. Similar studies using rat Leydig cells in culture demonstrated that treatment with hCG or cAMP increased the synthesis of P450scc and adrenodoxin *(102)*.

Studies by this author and colleagues using primary culture of mouse Leydig cells demonstrated that LH or cAMP are essential for the expression of P450c17 enzyme activity *(103,104)*, *de novo* synthesis of P450c17 protein *(105,106)*, and the expression of P450c17 mRNA *(107)*. The synthesis of P450c17 ceases in the absence of cAMP *(105)*. In a subsequent study, Youngblood and Payne identified the cAMP-responsive region between -346 and -245 bp upstream of the start site of transcription of the *Cyp17a1* promoter *(45)*. The essential role of cAMP for the expression of P450c17 in other species has been reviewed by Waterman and Keeney *(108)*. In contrast to the absolute requirement for cAMP for the expression of P450c17 in mouse Leydig cells, expression of P450scc and 3β-HSD are not dependent on cAMP *(105)*. Although LH, hCG, or cAMP may not be critical for expression of P450scc in Leydig cells in culture, treatment of mouse MA-10 Leydig tumor cells with cAMP *(106,109)* or forskolin *(110)* increases the amount of P450scc protein and mRNA.

In the studies on the requirement of cAMP for the expression of P450c17, evidence was obtained that testosterone produced during LH or cAMP stimulation repressed cAMP induction of P450c17 activity *(104)*, *de novo* synthesis *(106)*, and the amount of mRNA *(107)*. This negative effect of testosterone could be mimicked by the androgen agonist mibolerone *(107)* and prevented by the androgen antagonist hydroxyflutamide *(106)* indicating that androgen-mediated repression of P450c17 expression was mediated by the androgen receptor. A subsequent study demonstrated that androgen-mediated repression involved the binding of the androgen receptor to sequences within the cAMP-responsive region of the *Cyp17a1* promoter *(111)*.

Glucocorticoids have been implicated in the regulation of testicular steroidogenesis. Increased production of glucocorticoids in pathological conditions of the adrenal cortex, such as Cushing's syndrome, can be associated with decreased circulating testosterone and reproductive dysfunction *(112)*. Studies by Hales and Payne *(109)* and Payne and Sha *(107)* demonstrated that the glucocorticoids, cortisol, corticosterone, or the synthetic glucocorticoid, dexamethsone, repress both basal and cAMP-induced synthesis of P450scc protein and mRNA. The glucocorticoid-mediated decrease in P450scc synthesis was prevented by the antiglucocorticoid, RU486, suggesting that glucocorticoid repression of P450 synthesis is mediated by the glucocorticoid receptor found in Leydig cells *(113)*.

For regulation of P450c19 in Leydig cells, *see* Chapter 19.

Hydroxysteroid Dehydrogenases

3β-HSD

Gonadal expression of human 3β-HSD II and mouse 3β-HSD I is dependent on SF-1 as described for the gonadal-specific expression of the P450 steroidogenic enzymes *(114,115)*. A study on the mouse *Hsd3b1* promoter identified three potential SF-1 consensus binding sites in the proximal promoter of the gene *(75)*. In a subsequent study, it was shown that SF-1, also, was required for the expression of mouse 3β-HSD I protein *(76)*.

Studies involving the regulation of 3β-HSD mRNA in mouse Leydig cells in culture, demonstrated high constitutive expression of 3β-HSD *(107)*. In a subsequent study, it was found that mouse Leydig cells express two distinct isoforms of 3β-HSD, 3β-HSD I, and VI *(65)*. Investigations using gonadotropin-deficient mice to study the role of LH/hCG in regulating the expression of 3β-HSD I and VI mRNA in the adult Leydig cell lineage revealed that the expression of 3β-HSD I is independent of LH stimulation *(116)*. In contrast, the expression of 3β-HSD VI mRNA is highly dependent on LH/hCG stimulation. GATA factors appear to be important in the expression of human 3β-HSD II in steroidogenic cells *(100)*. In a recent study, Martin et al. identified a proximal element in the *HSD3B2* promoter that interacts with GATA 4 and 6 which physically interact with SF-1 or LHR-1 to determine cell-specific and maximal expression of *HSD3B2* in Leydig cells *(117)*. In addition, Martin and Tremblay *(118)* identified a response element located at -130 bp specific for another orphan nuclear receptor, Nur 77, which was found to be important for both basal- and

hormone-induced human *HSD3B2* promoter activity Nur 77 expression is induced in vivo by LH/hCG in the testis and appears to be an important mediator in the action of LH on steroidogenesis *(119)*.

17βHSD. Studies on the regulation of 17βHSD 3 are limited. Baker et al. *(120)* examined the expression of 17β-HSD 3 mRNA during development in normal mice, and mice lacking circulating gonadotropins (*hpg*), or functional androgen receptors (*Tmf*). The results showed that during neonatal development expression of 17β-HSD 3 mRNA is independent of gonadotropin action, but becomes dependent on androgen action at the time of puberty *(120)*.

5α-Steroid Reductase

During puberty in rat *(82)* and mouse *(83,84)* Leydig cells, 5α-steroid reductase is highly expressed resulting in 5α-androstanediol being the major androgen produced. Hypophysectomy of 21-d-old rats elicited a marked decrease in 5α-reductase activity *(121)*. Treatment of the rats with LH, initiated 6 d posthypophysectomy, resulted in a sharp increase in 5α-reductase activity. Treatment with FSH did not increase or prevent the decrease in 5α-reductase activity following hypophysectomy. Similarly, LH, but not FSH, increased 5α-reductase activity in *hpg* mice *(122,123)*.

Additional studies provided evidence that prolactin (PRL) is involved in the maintenance of high 5α-reductase activity in testes of immature mice *(124,125)*. PRL treatment of mice had no effect on 5α-reductase activity, but enhanced the LH-induced increase in activity *(125)*. Murono and Washburn tested several hormones in 25-d-old rat Leydig cells in culture and found only hCG increased 5α-reductase activity *(126)*. Additional studies by Murono et al. showed that the acidic fibroblast growth factor, as well as the platelet-derived growth factor repressed basal and hCG-stimulated 5α-reductase activity in cultured immature rat Leydig cells *(127)*. Basic fibroblast growth factor repressed hCG-stimulated 5α-reductase activity, but had no effect on basal activity *(128)*. In a subsequent study, Viger and Robaire examined the type and developmental expression of 5α-reductase mRNA and protein in the testis of rat *(129)*. At all ages examined, they identified type-1 5α-reductase in Leydig cells. Type-1 5α-reductase mRNA was found to the be most abundant in the immature rat between 21 and 28 d of age. Immunohistochemical staining with a specific antiserum to the type-1 enzyme localized the type-1 protein in the cytoplasm of Leydig cells with the highest expression between 21 and 28 d, followed by a progressive decrease closely paralleling the enzyme activity reported earlier by other investigators.

CLINICAL FEATURES OF MUTATIONS IN STEROIDOGENIC ENZYMES

P450 Enzymes

CYP11A1. Mutations, homozygous in the *CYP11A1* gene in human, are lethal *(130)*. Such mutations result in the inability of the placenta to produce progesterone, which is essential for maintenance of pregnancy beginning at 6–7 wk gestation when production of progesterone by the corpus luteum wanes.

CYP17A1. Numerous reports have been published describing patients with mutations in the *CYP17A1* gene. Mutations have been identified that cause either complete, partial, or isolated 17,20 lyase activity *(131–137)*. The patients exhibit a range of phenotypes depending on the mutation. Both male and female patients are hypertensive because of the overproduction of mineralocorticoids as well as impaired production of cortisol. Affected females exhibit abnormal sexual development resulting in primary amenorrhea. Male patients are phenotypic females due to the deficiency of testosterone production.

CYP19A1. To date five male patients with mutations in *CYP19A1* have been reported *(138–143)*. Defects observed in these patients are not because of the absence of aromatase activity in Leydig cells but, the absence of conversion of testosterone to estradiol in peripheral tissues. Male patients developed very high stature in their late twenties owing to the failure of epiphyseal fusion. Furthermore, they exhibited severely delayed bone age resulting in ostopenia and undermineralization. In addition, these patients experienced marked metabolic defects in carbohydrate and lipid metabolism.

Hydroxysteroid Dehydrogenases

HSD3B. Homozygous mutations in *HSD3B1* are lethal in human because the 3β-HSD I protein is required for progesterone synthesis in the placenta as described above for *CYP11A*. Many mutations in the *HSD3B2* gene have been identified and are summarized in a review by Simard et al. *(72)*. The major defect observed in males with mutations in the *HSD3B2* gene is either perineal hypospadias or perineoscrotal hypospadias and ambiguous external male genitalia or microphallus. These features in the male are because of decreased biosynthesis of testosterone that is required for normal development of external male genitalia *(72)*.

HSD17B3. Autosomal-recessive mutations in *HSD17B3* have been identified and characterized in numerous male patients *(144)*. The product of the *HSD17B3* gene is essential for Leydig cell biosynthesis

of testosterone during fetal development. Mutations result in phenotypic males with female external genitalia. These males have abdominal testes, epididymides, vas deferentia, seminal vesicles, and ejaculatory ducts. Plasma testosterone concentrations rise at the time of puberty resulting in many of these individuals undergoing marked virilization. The explanation for this phenomenon is most likely result of the peripheral conversion of circulating androstenedione by one of the other 17β-HSD isoforms *(145)*.

5α-Reductases

In males, SDR5A2 is expressed in external genitalia. Thus, mutations in *SDR5A2* result in various degrees of male pseudohermaphroditism with undermasculinized external genitalia (*see* Chapter 12).

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11 Development of Leydig Cell Steroidogenesis

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SUMMARY

During development two or three distinct populations of Leydig cells arise sequentially. These cell populations appear to represent distinct cell lineages and show clear differences in their regulation and pattern of development. The fetal Leydig cells arise soon after testis differentiation and appear to go through an early phase of hormone-independent development where paracrine factors or constitutive activity may regulate cell function. Thereafter, in the mouse, the cells become dependent on pituitary hormone action and current evidence suggests that both luteinizing hormone (LH) and adrenocorticotrophic hormone may regulate development. The adult population of Leydig cells arises before puberty, around day 7 in the mouse. Factors initiating and regulating this development are unclear but LH does not appear to play an early role in adult Leydig cell differentiation. Following differentiation, there is a marked proliferation of Leydig cell number before puberty although the cells do not become steroidogenically active until near puberty. Both proliferation and functional development of the adult Leydig cell population are critically dependent on LH although, in the mouse, androgens are also essential for normal Leydig cell development.

Key Words: ACTH; androgen; development; Leydig; LH; mouse; steroidogenesis; testis.

INTRODUCTION

In all mammalian species studied so far two or three population of Leydig cells arise sequentially during development, as discussed in Chapter 20. Two populations arise in the rodents and generally these are now accepted to represent two distinct cell lineages *(1–6)*. In the human it is reported that there are three phases of Leydig cell development *(7)*, although whether these represent distinct population remains to be determined. Both populations of cells exist primarily to secrete androgen although there is variation in the nature of steroids secreted and control mechanisms regulating cell function.

This chapter reviews our understanding of Leydig cell development with a particular focus on the mouse because of the availability of mouse models lacking specific hormones, growth factors, and receptors. These models are now providing us with good insight into the development and regulation of steroidogenesis in the different Leydig cell populations in the mouse, although, care must be taken if extrapolating to other species, as clear species-dependent variation exists.

FETAL LEYDIG CELL DEVELOPMENT

Fetal Leydig cells can first be identified morphologically around day 12.5 pc in the mouse and in the eighth week of gestation in the human *(8–10)*. As discussed elsewhere (Chapter 20), the origins of the fetal Leydig cell population remain uncertain but the stem cells are likely to arise from either the coelomic epithelium or the mesonephros *(11–13)*. Differentiation and development of the fetal Leydig cells is known to depend on a number of factors including steroidogenic factor (SF)-1, the Sertoli cell products Desert hedgehog, and platelet-derived growth factor-A, and the X-linked Aristaless-related homeobox gene (*Arx*), which is expressed in peritubular myoid cells, endothelial cells and interstitial fibroblasts.

Leydig cell steroidogenesis begins shortly after differentiation in the mouse although there is a gap of about 2 d in the rat between the first detection of

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3β-hydroxysteroid dehydrogenase (3β-HSD) activity and androgen synthesis *(14,15)*. In the human, testosterone can be detected in the fetal testis at 6–7 wk of gestation thus preceding apparent morphological differentiation *(16)*.

Early Induction of Steroidogenesis

The orphan nuclear receptor SF-1 acts to control expression of a large array of genes required for development and function of the component parts of the reproductive system *(17)*. During testis differentiation SF-1 is expressed in the developing Leydig cells and regulates expression of multiple genes encoding steroidogenic enzymes *(18–20)*. In mice with a Leydig cell-specific knockout of the gene that codes for SF-1 it has been shown that there is lack of *Cyp11a* and *StAR* expression, and it remains to be shown whether other genes are also affected *(21)*. Interestingly, it has been shown that likely multipotent stem cells, derived from bone marrow cells, will transform into steroidogenic cells when transfected with the gene that codes for SF-1 *(22)*. Thus, it is likely that early induction of Leydig cell differentiation and development is SF-1-dependent. Thereafter, it is likely that Leydig cells go through a hormone-independent stage of development, which may depend on paracrine regulation within the testis or on constitutive activity within the cells.

Evidence for a hormone-independent phase of fetal Leydig cell development is varied and comes from more than one species. Generally, initial testis and Leydig cell differentiation occur before gonadotroph development and gonadotrophin secretion in mammals *(23)* indicating that gonadotrophins are unlikely to be involved in testis differentiation. Other evidence comes from mice lacking a pituitary gland or the individual pituitary hormones like luteinizing hormone (LH), follicle-stimulating hormone (FSH), adrenocorticotrophic hormone (ACTH), GH, or prolactin. In these animals there is normal masculinization of the fetus *(24–30)* indicating the presence of functional Leydig cells during the crucial developmental period. In the human the placenta produces hCG before Leydig cell differentiation raising the possibility that this hormone may be involved in initial Leydig differentiation and function. This is not, however, supported by evidence from a human patient with an inactivating mutation in the LH/hCG receptor. In this individual there was some development of epididymis and ductus deferens indicating that hCG-independent androgen production occurred during early testis development *(31)*. In the rabbit, Leydig cell androgen production starts at about embryonic day 18. Studies in vitro using isolated rabbit testes have shown that this early androgen production appears to be independent of hormonal stimulation *(32)*.

Paracrine Regulation of Fetal Leydig Cell Steroidogenesis

A number of peptides have been implicated in development and function of the fetal Leydig cell population. Vasoactive intestinal peptide, pituitary adenylate cyclase-activating polypeptide, atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide all have been shown to have an acute stimulatory effect on androgen production by the fetal testis suggesting that they may act in a paracrine fashion to regulate fetal Leydig cell function *(33,34)*. This regulation may be of particular importance during the early phases of Leydig cell development, before hormonal regulation is established. Other local factors, which can regulate fetal Leydig cell function include retinoic acid and estrogen, which both act to inhibit steroidogenesis through effects on steroidogenic enzyme gene expression *(35,36)*. The extent to which these inhibitory factors regulate Leydig cell development under normal circumstances remains to be determined.

Hormonal Regulation of Fetal Leydig Cell Steroidogenesis

Mice null for the *thyroid-specific enhancer-binding protein* (*T/ebp*) gene lack a pituitary gland but undergo normal fetal masculinization, as discussed earlier *(27)*. Interestingly, however toward the end of fetal life testicular androgen levels are reduced more than 10-fold in these mice *(27)*. This indicates that as fetal Leydig cells develop through gestation they become dependent on pituitary hormones for normal function. Until relatively recently there was an assumption that mouse fetal Leydig cells would be LH-dependent during fetal development. However, studies using animals lacking circulating LH or the LH-receptor have shown that fetal Leydig cell function is normal in the absence of LH *(37,38)*. In mice lacking LH, testicular androgen levels are normal throughout fetal development and at birth, but decline thereafter, and are barely detectable by day 5 *(37)*. Thus, in the mouse, LH only becomes essential for Leydig cell function after birth. However, as fetal testicular androgen levels are significantly reduced in mice lacking a pituitary gland it is clear that a pituitary hormone, other than LH, is involved in regulation of fetal Leydig cell function. Subsequent studies have shown that ACTH will stimulate fetal Leydig cell function through direct action on the melanocortin type-2 receptor *(6)*. However, ACTH alone cannot be responsible for fetal androgen production as mice lacking ACTH

during fetal development show normal Leydig cell development *(6)*. Thus, fetal Leydig cells in the mouse are responsive to both LH and ACTH, but are not dependent on either hormone alone, suggesting that both hormones may act to regulate fetal Leydig cell development in a redundant fashion.

In contrast to the mouse, LH/hCG is crucial in the human for Leydig cell function during fetal development following the initial LH-independent phase. Beyond rudimentary development of the epididymis and ductus deferens masculinization does not occur in the absence of LH receptors *(31)*. However, it is uncertain, whether other hormones may also have an effect on fetal human Leydig cells, especially during the last trimester when hCG levels are lower, and during early postnatal development. There is evidence that chronic excessive ACTH levels in young boys can stimulate Leydig cell function leading to precocious puberty *(39,40)*, suggesting that the human fetal Leydig cell may also be sensitive to ACTH.

Steroidogenic Pathways

The steroidogenic enzymes and pathways involved in androgen synthesis by the Leydig cells are discussed in detail in Chapter 20. As the preferred pathway in a particular species is probably related to species-specific differences in the relative affinity of the 17,20-lyase reactions for Δ5 and Δ4 substrates the fetal and adult leydig cell populations, not surprisingly, utilize similar pathways to testosterone production *(41,42)*. However, one interesting difference between fetal and adult testis lies in the expression of 17β-hydroxysteroid dehydrogenase type-III (17β-HSDIII) which catalyses the final conversion of androstenedione to testosterone. In the adult testis this enzyme is expressed solely in the Leydig cells *(43),* whereas in the fetal testis, expression is predominantly in the tubules *(43)*. This implies that testosterone production in the fetal mouse testis requires co-operation between the interstitial and tubular compartments. Expression of 17β-HSDIII in the tubules may be an evolutionary remnant from when the Sertoli cell was the predominant steroidogenic cell in the testis *(44)*. Thus, during evolution of the Leydig cell the fetal population, in the mouse at least, retained a dependence on the tubules for androgen production, whereas the adult cells became fully independent.

Another difference in steroidogensis between the fetal and adult Leydig cells lies in the expression of "adrenal" enzymes by the fetal testis. Both CYP11B1 (11β-hydroxylase) and CYP21 (21-hydroxylase) are expressed in the fetal testis, but not the adult testis *(45,46)*. The physiological significance of the presence of these enzymes in the fetal testis, in terms of steroid production, is unclear, but their expression is further evidence of a developmental link between the fetal Leydig cells and cells of the adrenal cortex *(45,47)*.

ADULT LEYDIG CELL DEVELOPMENT

The adult Leydig cell population starts to develop in the mouse about postnatal day 7 *(4,48)*. Evidence that the adult population of cells is distinct from the fetal population comes from both morphological and functional studies, and includes differences in gene expression patterns between the two cell types *(1–4,6,49–51)*. The origin of the stem cells giving rise to the adult population of Leydig cells remains unknown. Surprisingly, it also remains unclear, which cells in the developing testis are the immediate progenitors of the adult Leydig cells. The consensus from most studies would indicate that the adult Leydig cells differentiate from peritubular mesenchymal cells *(52,53)*, although more recent study shows Leydig cells developing from vascular smooth muscle cells and pericytes *(54)*, and further studies are required to clarify this apparent contradiction.

Adult Leydig cell number in the mouse increases rapidly after initial differentiation and the final adult number of cells is reached between days 20 and 30 (Fig. 1).

Comparison of Leydig cell number and testicular androgen levels shows that the Leydig cells do not appear to become steroidogenically active until toward the end of this period Fig. 1 *(43,55,56)*. Circulating LH levels rise significantly between 20 and 30 d *(57)* but the cells appear to be largely insensitive to the effects of LH *(43,58)*. Measurement of the expression levels of the key genes involved in the steroidogenic function of the adult Leydig cells shows that, with the exception of the LH-receptor and 3β-HSD1 gene expression per Leydig cell is low at 20 d compared with the neonatal or adult testis (Fig. 2). This indicates that the newly formed adult Leydig cells are functionally immature and require to undergo a phase of maturation, which is regulated largely by LH (*see* following Subheading). These cells are likely to be analogous to the progenitor Leydig cell population, which have been more extensively characterized in the rat *(59)*.

Regulation of Adult Leydig Cell Development

It is clear that morphological and functional maturation of the adult Leydig cell population is critically dependent on LH stimulation. This is apparent from studies in mice lacking either circulating LH or the LH-receptor *(24,26,60–62)*. In these animals there is a

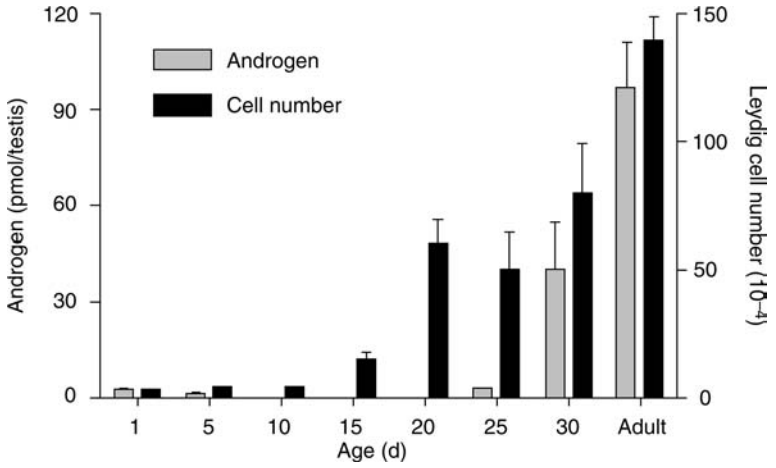


Fig. 1. Changes in testicular androgen levels and Leydig cell number during development in the mouse. Data show mean ± SEM of testicular androgen levels (black) and total Leydig cell number (gray) during development. Data are derived from refs. *56,63,* and unpublished data.

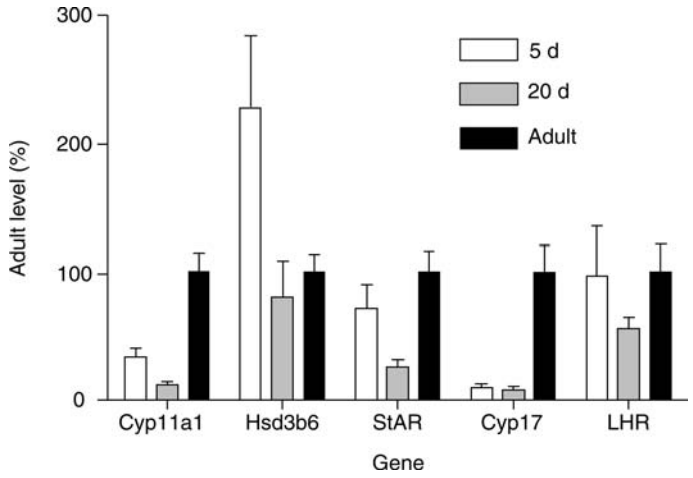


Fig. 2. Changes in gene expression per Leydig cell during postnatal development. Results show mean ± SEM of gene expression per testis corrected for the number of Leydig cells at each age. Cyp11a1, cytochrome-P450 side-chain cleavage; 3β-HSD1, 3β-hydroxysteroid dehydrogenase type-1; StAR, steroidogenic acute regulatory protein; Cyp17, cytochrome-P450 17α-hydroxylase; LHR, luteinizing hormone receptor. Data are derived from refs. *50,63*.

failure of normal adult Leydig cell development so that Leydig cell number in the adult animals are about 10% of normal *(63)* and androgen levels are very low or undetectable *(26,37,61)*. Despite the clear role for LH in proliferation and maturation of the adult Leydig cell population current evidence suggests that LH is not essential for the onset of adult Leydig cell differentiation. Initial, immunohistochemical studies showed that specific steroidogenic enzymes are detectable in rat adult Leydig cell precursors before LH-receptors are detectable *(52)*. More recently it has been shown that specific markers of the adult population of Leydig cells, such as 3β-HSD type-6 *(4)*, are expressed in Leydig cells from adult mice lacking circulating LH *(64)*. Therefore it appears likely that LH acts to induce adult Leydig cell proliferation and maturation but that, in the mouse at least, initial differentiation is LH-independent. The initial stimulus for adult Leydig cell differentiation remains unknown, although, thyroid stimulating hormone appears to be required *(65,66)*. In addition, the adult Leydig cell population fails to develop in mice lacking the Sertoli cell products Desert hedgehog and platelet-derived growth factor-A *(67,68)*, although, this may be related to a failure of stem cell development or proliferation.

Once Leydig cell differentiation has started there is an absolute requirement for LH stimulation in order to develop the steroidogenic potential of the cells. With

the exception of 3β-HSD type-1, expression of all key steroidogenic enzymes required for androgen biosynthesis is LH-dependent *(26,38,64)*. Expression of 3β-HSD1 does not appear to be under hormonal control and may be a constitutive component of Leydig cell differentiation. Recently, a novel stress-related gene termed *brain and reproductive organs expressed* has been reported to play a role in regulation of 3β-HSD1 *(69)*, although, whether this is of any normal developmental significance is unclear.

In animals lacking gonadotrophin stimulation there is good evidence that FSH can stimulate Leydig cell function through its effects on the Sertoli cell *(70,71)*. However, in animals with normal LH stimulation FSH does not appear to play a significant role in Leydig cell development or function *(72)*. Thus, any FSH-dependent factors, which act to regulate Leydig cell development appear to be redundant under normal conditions.

In contrast to FSH, androgens appear to be essential for normal Leydig cell development and function. In animals lacking the androgen receptor *Testicular feminized* (*Tfm*) Leydig cell number is reduced by about 40% and testicular androgen production is severely reduced *(50,73)*. This failure of androgen production is associated with loss of expression of several genes which are essential for normal Leydig cell function including *Cyp17*, *3β-HSD VI*, and *17β-hydroxysteroid dehydrogenase III (50)*. Initial expression of these genes is less markedly affected in *Tfm* mice during the period of early adult Leydig cell development indicating that normal Leydig cell differentiation occurs but that there is a failure of subsequent Leydig cell development *(50)*. Recent studies in mice which lack androgen receptors only in the Sertoli cells indicates that the major effect of androgen on Leydig cell development is mediated through direct action on the Leydig cells or, possibly, through the peritubular cells *(74)*.

CONCLUSION

This chapter outlines our current understanding of fetal and adult Leydig cell functional development. What has become clear in recent years is that the fetal Leydig cell population appears to arise and function, at least initially, without any hormonal input. It is also likely that initial differentiation of the adult Leydig cell population is gondotrophin-independent although these cells very rapidly become dependent on LH for further growth and differentiation. Initial independence from external regulation may be understandable given the essential role for these cells in development of the male phenotype and fertility. However, a number of uncertainties remain concerning Leydig cell development. In particular, the triggers, which initiate differentiation of the adult population of cells are unknown and identification of these factors would be a major step forward in our understanding of Leydig cell development and may allow specific manipulation of Leydig cell numbers in vivo.

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12 Peripheral Testosterone Metabolism

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SUMMARY

Testosterone is the principal androgen secreted by the human testis. Testosterone is carried by the circulation to target cells, where transactivation of the androgen receptor manifests its actions as a classical steroid hormone. Alternatively, the biological activity of testosterone might be increased, decreased, or categorically altered by enzymes in peripheral and target tissues. The 5α-reductase enzymes convert testosterone to more potent androgen, dihydrotestosterone (DHT), which is required for formation of the external genitalia in males. The biosynthesis of DHT also occurs in some species, including human beings, through an alternate pathway which does not utilize testosterone as an intermediate. Testosterone can be inactivated either through its conversion to androstenedione, mediated by 17β-hydroxysteroid dehydrogenase type 2, or through 5β-reduction. Testosterone, DHT, and their metabolites are subjected to glucuronidation and sulfation to facilitate excretion in the urine. Finally, some of the effects of testosterone are mediated through its aromatization to the potent estrogen, estradiol. This chapter discusses the peripheral metabolic pathways of testosterone, the key enzymes involved, and their physiology and genetics.

Key Words: Androgens; dihydrotestosterone; 5α-reductase; 17β-hydroxysteroid dehydrogenase; testosterone.

INTRODUCTION

The principal androgen secreted by the human testis during fetal and adult life is testosterone (T). Like all hormones, T is carried through the bloodstream to peripheral sites of action. However, T can also undergo reversible and irreversible metabolism to other steroids with different activities, and T might also derive from metabolism of 19-carbon adrenal precursors. This chapter will review the various metabolic fates of androgens in the peripheral tissues, the enzymes, which catalyze these reactions, and the general principles that govern androgen metabolism in peripheral tissues.

TESTOSTERONE SECRETION, DISTRIBUTION, AND DISPOSITION

The testis secretes from 3 to 10 mg/d of T and contributes more than 95% of total circulating T in the postpubertal human male *(1)*. Leydig cell-derived T diffuses into the circulation down a concentration gradient, where it equilibrates between protein-bound (98%)- and free hormone (2%) fractions, with the free hormone fraction generally, believed to be the biologically active form *(1)*. The majority of protein-bound T (~54% of total T) is bound to low affinity, high abundance proteins (primarily albumin), whereas the remainder of protein-bound T (~44% of total T) is bound to the high affinity, low abundance glycoprotein sex hormone-binding globulin *(2)*. The free T, which exists in rapid equilibrium with albumin-bound T, diffuses from the intravascular space into the interstitium and then to target tissues. In target cells, T can directly mediate either genomic changes by binding to the androgen receptor (AR), or nongenomic changes, which can involve either AR or other second messenger pathways. Alternatively, T can exert actions upon target cells following its conversion to other active steroid hormones, principally, either 5α-(DHT) dihydrotestosterone or 17β-(E2)estradiol, catalyzed by 5α-reductases and aromatase, respectively. Finally, T can be inactivated by its oxidation to androstenedione or its 5β-reduction to 5β-DHT. Combinations of metabolic pathways convert the majority of T and DHT to inactive 19-carbon byproducts, including (5α-)androsterone, (5β-)etiocholanolone, and the 5α- and 5β-androstane-17β-diols, which are glucuronidated or

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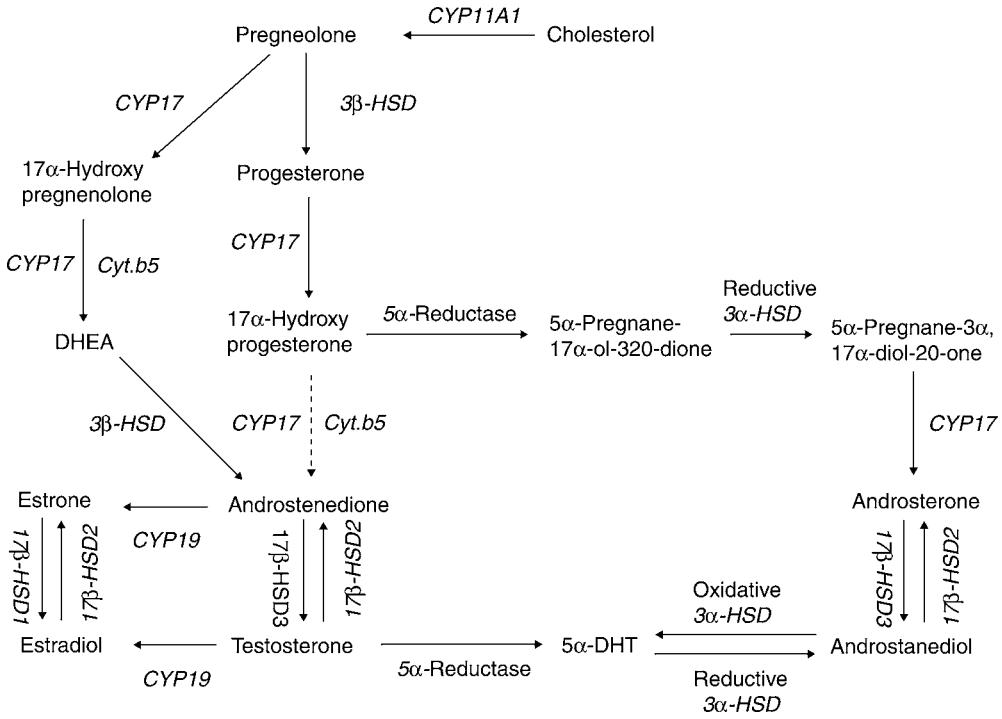


Fig. 1. Major pathways of DHT synthesis and metabolism. The major pathway involves the synthesis of dehydroepiandrosteron (DHEA), followed by its conversion to androstenedione and testosterone. In the minor pathway, 17-hydroxyprogesterone is converted to androstenedione directly. Testosterone can be 5α-reduced to DHT, and both androstenedione and testosterone can be aromatized to estrone and estradiol, respectively. In the alternate or "backdoor" pathway, 5α-pregnane-3α,17α-diol-20-one is cleaved to androsterone, which is sequentially converted to androstanediol and then DHT.

sulfated and excreted in urine. The major pathways of DHT synthesis and metabolism are summarized in Fig. 1.

MAJOR TESTOSTERONE-METABOLIZING ENZYMES

The 5α-Reductases

In contrast to most other classical steroid hormones, many of the most important functions of T are mediated through its conversion to the 5α-reduced steroid DHT *(3)*. Although, DHT was previously recognized as a T metabolite, it was not until Wilson and colleagues *(4)* demonstrated that DHT accumulates in nuclei of cells in the ventral prostate of rats, treated with radiolabeled testosterone, the 5α-reduction of T was recognized to be more than just a catabolic pathway for T. Subsequent studies demonstrated that conversion of T to DHT in the genital tissues of both human *(5)*, and lower mammals *(6)* occurs during the differentiation of these tissues into the male external genitalia—prostate, penis, and scrotum. In addition to its autocrine effects within classical androgen target tissues, DHT circulates in plasma at 10% of the concentration of testosterone, acting likely as a classical hormone *(7)*. In contrast, T itself appears to mediate the differentiation of the Wolffian ducts into the male internal genitalia—epididymis, vas deferens, and seminal vesicle.

The significance of the T to DHT conversion by 5α-reductase is due at least in part to the differential affinity of the AR for these two substrates. DHT is 10 times more potent than testosterone at inducing transcription of AR-driven reporter constructs, an observation explained by the 10-fold higher affinity of the AR for DHT, which primarily derives from the slower dissociation rate of DHT *(8)*. However, circulating T concentrations in fetal life are sufficient to saturate the AR in many tissues, so affinities alone do not explain the different actions of T and DHT. AR actions require the recruitment and dissociation of coactivators and corepressors, respectively, in order to interact with the basal transcriptional machinery *(9)*. The binding of T and DHT to AR results in different complexes of AR and other effector proteins in various target tissues, which in part explains the different actions of T and DHT *(10)*.

The importance of DHT in human physiology was confirmed with a study of the syndrome known as pseudovaginal perineoscrotal hypospadias, an autosomal recessive form of male pseudohermaphroditism. Affected individuals are karyotypic males (46, XY) with male internal genitalia, including testes, epidydimides, vasa deferentia, and seminal vesicles, but exhibit female-appearing external genitalia. Prepubertal patients with this disorder usually, have a blind vaginal pouch, a phallus that resembles a clitoris, hypospadias, and incompletely descended testes *(11)*. At puberty, serum T level rises, whereas DHT remains low, resulting in enlargement of the phallus, increased muscle mass, and descent of the testes into the labioscrotal folds, normal amounts of acne, but without absence of facial and body hair, or lack of temporal balding *(12)*. Most postpubertal patients exhibit male psychosexual orientation, resulting in a reversal of gender identity of individuals who were raised as females *(13)*. Genital skin cells from affected individuals have a reduced capacity to convert T to DHT, and affected subjects exhibit a decreased capacity to 5α-reduce infused testosterone. Both observations are consistent with impairment of the 5α-reductase enzyme responsible for the conversion of T to DHT in genital skin *(14,15)*, so this syndrome was renamed 5α-reductase deficiency.

In the late 1980s, David Russell and colleagues *(16)* circumvented the difficulty of purifying the hydrophobic, membrane-bound 5α-reductase by designing an oocyte expression-cloning strategy to isolate a rat complementary DNA encoding a 5α-reductase enzyme with a predicted molecular weight of 29,000. A human cDNA was subsequently isolated (5α-reductase type 1), and study of the recombinant protein expressed from this cDNA revealed an enzyme with a broad, alkaline pH optimum in contrast to the sharp, acidic pH optimum typically exhibited by the 5α-reductase activity in prostate homogenates *(17)*. Furthermore, the gene encoding the 5α-reductase type 1 isoenzyme was found to be normal in individuals with 5α-reductase deficiency *(18)*. These observations led to the cloning of a second cDNA for 5α-reductase type 2, and deletions or mutations were subsequently found in the gene for this isoenzyme, in subjects with 5α-reductase deficiency *(19)*. Genetic deficiency of 5α-reductase type 1 has yet to be demonstrated, so the role(s) of the type 1 isoenzyme in human physiology remains unknown.

In addition to complete deletions, a variety of point mutations have been identified in the gene for 5α-reductase type 2 in patients with 5α-reductase deficiency. These include mutations that impair either NADPH binding (which cluster at the 5′ and 3′ ends of the gene) or T binding (which cluster in the 3′ half of the gene), as well as mutations that produce truncated enzymes or enzymes with reduced stability *(3,20)*.

The human 5α-reductase isoenzymes share 50% amino acid sequence identity, but neither enzyme can be classified into any previously known enzyme family. All known 5α-reductases contain about 250 amino acids, with calculated molecular weights ranging from 21,000 to 29,000 *(3)*. The 5α-reductases are NADPH-dependent enzymes that irreversibly reduce the $\Delta^{4,5}$ double bond of 3-keto, Δ^4-steroids to produce 5α-reduced, 3-ketosteroids. Although, the human cDNAs for these enzymes were isolated based on their capacity to reduce T to DHT, both isoenzymes, particularly the type 1, show superior catalytic activity with 21-carbon steroids such as progesterone and 4-pregnene-20α-ol-3-one. The structural motifs responsible for NADPH binding to the 5α-reductases are not known, as these enzymes lack any region of homology to the well-characterized NADPH-binding domains, such as the Rossman fold of short-chain oxidoreductases (SCOR) *(3)*. The apparent K_m of T is 1–5 μ*M* for the type 1 isoenzyme, but only 0.1–1 μ*M* for the type 2 isoenzyme *(3)*. The type 2 isoenzyme is more potently inhibited by finasteride than is the type 1 isoenzyme, whereas 17β-(*N*,*N*′-diethyl)-carbamoyl-4-methyl-4-aza-5α-androstan-3-one (4-MA) and dutasteride inhibit both isoenzymes with comparable affinities *(3)*.

In humans, both 5α-reductase isoenzymes are expressed in liver following birth, but the type 2 isoenzyme is the predominant form in genital skin and prostate, an expression pattern that is consistent with the phenotype seen in human 5α-reductase type 2 deficiency. Both isoenzymes are also expressed in nongenital skin from birth to 2–3 yr of age and also from puberty onward. It is likely that the conversion of T to DHT by the type 1 isoenzyme in liver and skin provides the source of DHT that enables virilization at puberty in 5α-reductase type 2 deficiency *(21)*.

Rats exhibit a unique, sexually dimorphic expression of the type 1 isoenzyme, with 20-fold higher expression in the livers of females than in males *(21)*. Knockout mouse technology has been used to study the roles of the types 1 and 2 isoenzymes in mice by deleting the two genes for these proteins singly and in combination. Targeted deletion of the type 1 isoenzyme in mice causes reduced fecundity and delayed parturition in homozygous null females, although, the mechanism for this phenotype is not understood. The parturition defect could be secondary to enhanced

Table 1
Major Enzymes of Peripheral Testosterone Metabolism

Enzyme	*Major tissues*	*Major reaction*	*Cofactor*	*Human deficiency*
5α-reductase 1	Liver, skin	T to DHT	NADPH	None identified
5α-reductase 2	Prostate, genital skin, liver	T to DHT	NADPH	Male pseudohermaphroditism
17β-HSD2	Liver, small intestine, placenta, prostate	T to A		None identified
AKR1C4	Liver	DHT to Adiol	NADPH	None identified
AKR1C2 and -1C3	Prostate	DHT to Adiol	NADPH	None identified
RODH (3[α→β]HSE)	Prostate	Adiol to DHT	NAD^+	None identified

HSE, hydroxysteroid epimerase.

aromatization of T in the face of impaired conversion to DHT, or secondary to impaired 5α-reduction of progesterone *(22)*. The biological role of 5α-reductase type 1, thus, might be to regulate the balance among androgen, estrogen, and progesterone at appropriate times in the reproductive cycle. Type 1 homozygous null males are phenotypically normal, suggesting that the type 1 5α-reductase has limited physiological importance in the male *(23)*. In contrast to humans, male mice lacking either 5α-reductase 2 alone or both 5α-reductases 1 and 2 exhibit only a mild impairment in virilization of the external genitalia despite undetectable plasma DHT concentrations, implying that male genital differentiation in mice is minimally dependent upon DHT *(23)*.

The principal androgen secreted by human testes during fetal, neonatal, and adult life is T, which is converted to DHT in the periphery. In contrast, the principal steroid secreted by testes in some species, including neonatal rodents and hamsters, as well as tammar wallaby pouch young, is 5α-(Adiol) androstane-3α,17β-diol. Adiol is oxidized by 3α-(HSDs) hydroxysteroid dehydrogenases in target tissues such as prostate *(24)* and is thus an efficient precursor to DHT. This Adiol sometimes derives from an alternative or "backdoor" pathway that bypasses T and occurs by initial 5α-reduction and 3α-reduction of progesterone or 17α-hydroxyprogesterone, which is mediated by 5α-reductase type 1 in the mouse. The 17-hydroxylated intermediate, 5α-pregnane-3α,17α-diol-20-one, is an excellent substrate for the 17,20-lyase activity of CYP17, yielding the 19-carbon, 17-ketosteroid androsterone, which is reduced to Adiol *(25)*. Although, a role of this pathway in normal human sexual differentiation has not been demonstrated, emerging evidence suggests that this pathway contributes to virilization of females in states of 17-hydroxyprogesterone accumulation. It remains an intriguing possibility that defects in sexual differentiation such as idiopathic hypospadias might be explained by polymorphisms/mutations in either the oxidative or the reductive 3α-HSDs necessary for the function of this pathway.

The HSDs

HSDs regulate intracellular steroid hormone potency by interconverting steroid pairs between their active and inactive forms. This interconversion occurs through the nicotinamide cofactor-dependent oxidation/reduction of steroids at discrete positions on the steroid nucleus. HSDs that participate in androgen metabolism include the 3α-HSDs, 3β-HSDs, and the 17β-HSDs, which are named according to the position on the steroid nucleus in which oxidation/reduction occurs (Table 1). Intracellular control of AR occupancy and activation is regulated by the activities of the 17β-HSDs and 3α-HSDs for which T and DHT are substrates.

Generally, HSDs can be classified into two structurally distinct enzyme families: the short-chain oxidoreductases and the aldo-keto reductases (AKRs) *(26,27)*. The SCOR enzymes are characterized by a YXXXK catalytic motif (where Y is tyrosine and K is lysine) and a glycine-rich nicotinamide-binding motif with a β-α-β structure known as a Rossman fold. HSDs in the SCOR class exhibit high catalytic efficiency and substrate selectivity, and most are tightly membrane-bound. In contrast, the AKRs are soluble enzymes with slower catalytic rates than SCOR-type HSDs. AKRs exhibit a structural fold known as a triosephosphate isomerase enzyme barrel (named after the prototypical member of this family), with catalytic sites containing tyrosine and lysine residues that are distant from each other in primary sequence *(28)*.

Multiple 17β-HSD isoforms exist in humans, and each isoenzyme is the product of a distinct gene *(29)*. These isoforms each metabolize a characteristic repertoire

of steroid substrates, which might include androgens, estrogens, and their precursors. The androgen-metabolizing 17β-HSDs include the SCOR types 2 and 3 enzymes and 17β-HSD5 (formally AKR1C3). The human genome contains four *AKR1C* genes in tandem on chromosome 10 *(30)*. Each one of the enzymes encoded by these *AKR1C* genes have 17β-HSD (and 3α-HSD) activities with certain substrates *(31)*. Finally, other enzymes in the SCOR family show 3α-HSD activity, and these enzymes also have retinol dehydrogenase activity (RoDH) *(32)*. The most important of these enzymes, which is found in prostate, is simply known as RODH *(24)*.

In addition to steroid substrate selectivity, each HSD shows a prominent directional preference for oxidation or reduction of steroid when the cDNA for the enzyme is expressed and assayed in intact cells. For example, 17β-HSD3 completes the synthesis of T by efficiently reducing androstenedione (A) to T in the Leydig cell, whereas 17β-HSD2 oxidizes T almost completely to A in peripheral tissues and transfected cells *(33)*. However, dual-isotope scrambling experiments using intact cells reveal that both 17β-HSD2 and 17β-HSD3 catalyze both the oxidation and reduction of A and T, establishing a pseudoequilibrium in which the rates of oxidation and reduction are equal *(34)*. The pseudoequilibrium set point established by a given HSD isoform lies either primarily toward T for the "reductive" enzyme 17β-HSD3 and primarily toward A for the "oxidative" enzyme 17β-HSD2. Similar directional preferences are observed for the 3α-HSDs, with the AKR1C enzymes demonstrating a strong reductive preference and RODH having a strong oxidative preference in intact cells. This directional preference is critical, because the magnitude of this preference determines the extent of AR transactivation within a given cell.

Because the HSD reactions are fundamentally reversible equilibria, the factor which governs whether the directional preference of a given HSD is oxidative or reductive is primarily not its relative affinities for steroid substrates, but rather the enzyme's relative affinities for the various forms of nicotinamide cofactors *(35)*. For example, 17β-HSD3 binds the NADPH/$NADP^+$ cofactor pair with high affinity. In the cytoplasm of healthy cells with adequate glucose, the pentose phosphate pathway, and cytoplasmic isocitrate dehydrogenase maintain a high NADPH/$NADP^+$ gradient. As a result, the high NADPH/$NADP^+$ gradient, as well as the thermodynamic drive of NADPH oxidation (~30 kcal/mol), drive the 17β-HSD3 equilibrium in the reductive direction *(35)*. In contrast, 17β-HSD2 binds the NADPH/$NADP^+$ cofactor pair with poor affinity, but binds the NAD^+/NADH cofactor pair with high affinity *(35)*. In the cytoplasm of well-oxygenated cells, a high NAD^+/NADH gradient is maintained by oxidation of NADH and oxygen reduction through the mitochondrial electron transport chain and oxidative phosphorylation. Consequently, the high NAD^+/NADH concentration gradient drives T oxidation by mass action despite the unfavorable energetics of NAD^+ reduction, with reoxidation of NADH providing the ultimate thermodynamic drive *(35)*.

The observation that HSD directional preference depends on cofactor affinity and cofactor gradients in intact cells leads to the prediction that, changes in either cofactor affinity or cofactor gradients could alter the intracellular content of active steroid hormones. Such changes could occur through allelic variants in the HSD genes, which alter cofactor affinities as well as through metabolic disturbances that modify cofactor gradients. This hypothesis has been validated in model systems using both site-directed mutagenesis to alter relative cofactor affinities of SCOR *(34)*, and AKR *(36)* HSDs, and glucose deprivation, which manipulates cellular redox state *(36)*. Both of these alterations change HSD pseudoequilibrium set points, converting the strong reductive preference of 17β-HSD1 *(34)* or AKR1C9 *(36)* to weakly reductive or frankly oxidative.

17β-HSD Type 2

The conversion of T to A and DHT to 5α-androstanedione by 17β-HSD2 serves a crucial role in regulating intracellular androgen concentrations and activation of the AR in peripheral tissues. Human 17β-HSD2 is a 42 kDa microsomal enzyme with an endoplasmic reticulum-retention signal predicted to direct its active site toward the strongly oxidizing environment of the endoplasmic reticulum lumen *(37)*. Human 17β-HSD2, which was originally cloned from a prostate cDNA library, oxidizes the 17-hydroxyl groups of several androgens, including T and DHT, as well as 17β-E2 to produce the inactive 17-ketosteroids, principally A and estrone. The apparent *Km* values for either testosterone or 17β-E2 as substrate are in the submicromolar range *(37)* and turnover is efficient, suggesting that androgens and estrogens are major physiological substrates for the enzyme. Interestingly, 17β-HSD2 also exhibits a 20α-HSD activity, oxidizing the inactive 20α-dihydroprogesterone to the active progestin, progesterone. The gene encoding 17β-HSD2, which is located on chromosome 16q24 *(38)*, is expressed in liver, small intestine, and placenta, and to a lesser extent in kidney, colon, pancreas, and prostate. The activity and expression pattern of 17β-HSD2 is

consistent with its physiologic role as an inactivator of androgens and estrogens in peripheral tissues *(38)*.

Complete deficiency of 17β-HSD2 has not been described in humans, so the presumed role of this enzyme in human physiology remains uncertain. The importance of 17β-HSD2 as an inactivator of androgens is suggested by studies demonstrating reduced 17β-HSD2 expression in prostate cancer tissues *(39,40)*. Indeed, clinically aggressive prostate cancers have been associated with loss of heterozygosity at 16q24.1-16q24.2, the chromosomal location of 17β-HSD2 *(41)*. Expression of 17β-HSD2 is induced in endometrium by progesterone, which enhances the conversion of 17β-E2 to estrone *(38)*. Decreased expression of 17β-HSD2 has been found in both endometriosis tissue and in colon cancer *(42,43)*, which are states, in which the effects of 17β-E2 appear to be potentiated. These data suggest a causative role of reduced 17β-HSD2 expression in the pathogenesis of disorders of prostate and endometrial growth. Recently, a 17β-HSD2 knockout mouse has been generated by homologous recombination, and homozygous null animals exhibit unexpected anomalies, including hydrocephalus, polycystic kidneys, and a variety of other dysmorphic features *(44)*. These observations suggest more complex roles of 17β-HSD2 beyond androgen and estrogen inactivation, at least in the mouse.

3α-HSDs

Many of the human enzymes catalyzing 3α-reduction of steroid substrates fall within the AKR1C family of enzymes, which together cluster under the larger AKR superfamily. Four major isoforms exist in humans, AKR1C1-AKR1C4, each of which possess a characteristic repertoire of 3α-, 17α-, and 20α-HSD activities with various substrates *(31)*. These enzymes accomplish their remarkable regiochemical diversity by binding steroid substrates in multiple orientations *(45)*. AKR1C4 is highly expressed in liver and efficiently reduces 5α-DHT and 5β-DHT at position 3 on the steroid nucleus, thus, promoting the catabolism and excretion of testosterone. AKR1C3 and AKR1C2 within the prostate serve to 3α-reduce DHT to Adiol, decreasing the amount of DHT available for AR binding in these cells *(31)*. Within the same tissue, membrane-bound SCOR-type HSDs within the RoDH subfamily oxidize Adiol to DHT. The principal oxidative 3α-HSD in prostate, named RODH, also has 3β-HSD activity, converting 3-keto products to the epimeric alcohols (i.e., 3β-Adiol) *(46)*. Consequently, RODH is also known as "3(α→β)hydroxysteroid epimerase" (3[α→β]HSE); although, neither the mechanism of this bifunctional catalysis nor the physiologic role has been elucidated.

ANDROGEN CATABOLISM

The principal route of clearance of T from the body is through urinary excretion. Phase 1 metabolism consists of catalytic transformations of the steroid ring structure, beginning with 5α- and 5β-reductases in liver irreversibly converting T to 5α-DHT *(17)* and 5β-DHT *(47)*. The 3α-reduction of each DHT epimer, followed by 17β-oxidation of each resultant product produces androsterone and etiocholanolone, respectively, which are the most abundant T metabolites *(48)*. Other transformations include oxidation, reduction, and hydroxylation reactions at other positions on the steroid ring structure, which yield numerous less abundant T metabolites *(48)*. These steroids then undergo the conjugative phase II reactions, primarily glucuronidation and sulfation, in liver, kidney, and even target tissues such as prostate to produce polar conjugates that are readily excreted in urine. Only about 2% of T is excreted as T itself, with the vast majority being first metabolized to roughly equal amounts of androsterone and etiocholanolone conjugates.

CONVERSION TO ESTROGENS: AROMATASE

Some physiological effects that accompany normal T synthesis are mediated neither by T nor by DHT, but by estrogens derived from androgen precursors. In the human male, roughly 0.2% of circulating T is converted to the potent estrogen E2 in peripheral tissues *(1)*, which accounts for almost 40% of the total daily E2 production in males. The remainder of E2 is derived either directly from the aromatization of T in the testis itself, or from conversion of adrenal precursors *(2)*. Conversion of T to E2 is catalyzed by aromatase (CYP19), a microsomal P450, which is expressed in a variety of tissues, including the gonads, brain, placenta, and adipose stromal cells. The rare syndromes of estrogen insensitivity and aromatase deficiency are associated with delayed epiphyseal closure and osteopenia *(49)*, demonstrating that most gonadal steroid influence on skeletal maturation—even in males—is mediated by estrogens. Conversely, increased aromatization, as a result of rearrangements in the promoter for the aromatase gene, has been reported to cause gynecomastia in males *(50)*.

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13 Leydig Cell Aromatase

From Gene to Physiology

Serge Carreau, PhD

SUMMARY

The ability of the mammalian testis to produce estrogens is well known and Leydig cells represent an important source. Taking into account the widespread distribution of estrogen receptors in male gonad and additionnal expression of aromatase in germ cells, the role of estrogens in male reproduction is more complex than previously realized.

Key Words: Aromatase; estrogen receptors; estrogens; germ cells; Leydig cells; mammals; reproduction.

INTRODUCTION

In mammalian testes gonadotropins and testosterone together with numerous intratesticular modulators are responsible for the induction and/or maintenance of spermatogenesis *(1,2)*. Estrogens have been for a long time considered as a specific female hormone; however, the presence of estrogens in the male gonad has been well-documented since the publication of Zondek more than 70 yr ago *(3)* showing the presence of estrogen in stallion urine. Numerous studies have then confirmed that the testis is a source of estradiol *(4–9)*. Indeed, in the rete testis fluid and in the caput epididymis of vertebrates, estrogen concentrations are more than in the blood *(10)*. For instance in man, the concentration of estrogens in the spermatic vein is 50-fold higher than in the peripheral plasma *(11)*, indicating a testicular origin for these hormones. Cytochrome P450 aromatase (P450arom) is a terminal enzyme present in endoplasmic reticulum of numerous tissues, including testis, which irreversibly transforms androgens into estrogens in males. The ability of the testis to synthesize estrogens has been intensively re-examined over the last 10 yr as the sequence of the aromatase gene became known *(2,12)*. Until recently the role of estrogens in male fertility was still the subject of debates, although a growing body of evidences favored a positive role.

LEYDIG CELL AROMATASE

What is Known About Aromatase Enzyme Activity, Immunohistolocalization, and Specific Transcripts

Despite many experiments realized both in vitro and in vivo, in different species of various strains, an agreement on the precise localization of aromatase activity at different ages has not always been evident (Table 1). Indeed, studies investigating the testicular site of aromatization of androgens in rat testicular tissues and/or in isolated cells have shown that Leydig cells express aromatase and furthermore, that aromatase activity is acutely stimulated by LH/human choriogonadotropin in vitro *(13–16)*. In the rat there is an age-related change in the cellular distribution of aromatization, being mainly in Sertoli cells in immature animals and switching to Leydig cells in adults *(16–19)*. With the availability of antibodies, aromatase has been immunohistolocalized in Leydig cells of young and adult rats *(20,21)*. Some of the variation observed between immunohistolocalization and enzyme activity in cell culture, could result from an absence of endocrine and/or paracrine regulation *(22)*, such as after in vivo germ cell depletion or lack of cell–cell contacts in culture dishes *(19,23)*. The Leydig and Sertoli cells of the rat express the gene for aromatase at all ages *(24,25)*.

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Table 1
Cytochrome P450 Aromatase (mRNA, Protein, and Activity) in Leydig Cells of Adult Mammals

Species	*mRNA*	*Protein*	*Activity*
Mouse	+ *(13)*	+ *(13,14)*	+ *(13)*
Rat	+ *(15,18,19)*	+ *(15–17,20)*	+ *(18,19,21–26)*
Bank vole	+ *(27,28)*	+ *(27,28)*	+ *(27,28)*
Squirrel	ND	ND	+/– *(29)*
Boar	+ *(35,37)*	+ *(35,36,38)*	+ *(30–35,37)*
Ram	ND	ND	+ *(39)*
Stallion	ND	+ *(40–42)*	+ *(40)*
Bear	ND	+ *(43)*	+ *(43,44)*
Monkey	ND	ND	+ *(45)*
Human	+ *(49)*	+ *(48,50)*	+ *(46–50)*

+, positive; –, negative; ND, not determined.
mRNA: P450 aromatase transcripts.
Protein: revealed by immunohistolocalization and/or Western blot.
Enzyme activity: measurement of estradiol output by RIA and/or determination of enzyme activity.
Main references are given in parentheses.

Although, in Leydig cells the level of P450arom mRNA is of the same magnitude whatever the age *(24)*, aromatase enzyme activity increases with age from day 20 onwards *(17)*. Conversely in Sertoli cells of adult rats the amount of P450arom is 20-fold lower compared with that of 20 d-old rats following trend of enzyme activity in parellel *(16,17)*. In adult rats, the low level of P450arom gene expression in Sertoli cells is related to negative control exerted by the neighboring germ cells *(26)*. In mice *(27)* and bank voles *(28)* Leydig cells are the source of estrogens. In seasonally breeding rodents, the P450arom (transcript, activity, and immunolocalization) was found to be related to photoperiod being elevated in species that breed in long day–light cycles rather than during the winter season *(28,29)*. In boars all data available are in agreement that Leydig cells represent the only source of estrogens *(30–33)*. This was confirmed recently in the minipig *(34)*. The stallion was the first mammal, in which estrogens have been discovered to be produced by the testis *(3,5)*. Later data have confirmed that the testicular source of estrogens in horse resides exclusively in the Leydig cells *(35–37)*.

In the human testis in addition to the report of Jayle et al. *(8)* showing production of phenolsteroids, the data of Payne et al. *(38)* clearly demonstrated that the interstitial tissue is the main source of estrogens. Later on, in vitro studies performed on purified Leydig cells *(39)*, and immunolocalization of aromatase *(40,41)* confirmed that human Leydig cells synthesize and secrete estrogens. Specific mRNA transcripts for aromatase have also been detected in human Leydig cells *(42)*.

Regulation of Aromatase

Aromatase is made up of two proteins: a ubiquitous NADPH-cytochrome P450 reductase and a cytochrome P450 aromatase, which contains the heme and the steroid binding pocket. In human, P450arom is the product of a single gene, Cyp19, located on chromosome 15, which belongs to the cytochrome P450 gene family, containing more than 500 members (*see also* Chapter 10). The *Cyp19* gene lies on more than 120 kb length with a coding region of 9 exons + 9 untranslated exons I. Analysis of transcript and genomic sequences indicates that *Cyp19* gene expression is regulated by multiple tissue-specific promoters producing alternate 5′-untranslated forms of exon I that are then spliced onto a common 3′-splice acceptor site in exon II upstream of the translation start site *(43–44)*. Despite this generation of *Cyp19* variants with different 5′ UTRs, the coding sequences are identical and give rise to a unique protein of 503 amino acids in human with a mol wt of 55 kDa.

The aromatase complex is located in the endoplasmic reticulum of cells. The promoter II (PII) proximal to the translation start site regulates P450arom expression in mammalian gonads *(45–46)* as well as in Leydig cell tumors *(47)*. In testis, FSH and LH both act to increase concentrations of intracellular cyclic AMP thereby inducing expression of P450arom, which in turn requires the transcription factors, cAMP response

element binding protein (CREB), cAMP response element modulator, and steroidogenic factor (SF)-1. SF-1 belongs to the nuclear orphan receptor superfamily and regulates steroidogenic genes transcription among them P450arom through its interaction with numerous coactivators, for example, CREB binding protein, DAX-1, SOX-9, WT1. Indeed, in mature rat Leydig cells, the addition of either LH (or dbcAMP) or testosterone induced a dose-related increase of P450arom transcripts *(48,49)*. Using RACE-PCR it has been shown that PII is the main promoter driving aromatase gene expression in primary Leydig cells *(50)*, which agrees with data obtained using Leydig cell lines *(47,51,52)*. Moreover, when purified Leydig cells were incubated with increasing concentrations of Sertoli cell-conditioned medium, a dose-related increase of the *Cyp19* gene expression was observed *(48)*. Consequently in order to analyze the putative role of factors that are likely to be present in testis, such as nuclear transcription factor (SF-1 or the liver receptor homolog LRH-1, an orphan receptor closely related to SF-1), purified testicular cells from mature rat have been prepared. It has been shown that LRH-1 is present both in Leydig cells and germ cells, but not in Sertoli cells, which by contrast express SF-1, and that LRH-1 increases P450arom gene expression in Leydig TM3 cell line *(53)*. Numerous other factors modulate aromatase activity in the rat *(54,55)*, and bank vole *(56)* as well as in isolated pig Leydig cells *(34,57)*. It has also been also shown that the level of P450arom mRNA is increased in Leydig cells of mice deficient in DAX-1 expression *(58)*.

In human gonads P450arom is also known to be regulated through the proximal promoter PII *(59)*. Taking into account that SF-1 and DAX-9 are playing an important role in sexual differentiation, testicular development and function, it is therefore obvious that fine tuning regulation of gonadal aromatase levels through PII is critical *(60)*. Aberrant expression of aromatase has been reported in testicular tumors (leydigoma or seminoma) leading to increased estradiol production which is deleterious for spermatogenesis *(61,62)*.

OTHER SOURCES OF ESTROGENS IN THE MALE GONAD

As abundantly documented in the literature, it is difficult to find a tissue completely devoid of aromatase gene expression *(43)*, but in some mammalian species such as pig *(32)* and human *(40)*, a P450arom mRNA was thought to be present only in Leydig cells. However, striking species differences exist because in mouse *(27)* and bear *(63,64)*, for example, the P450arom is present not only in Leydig cells but also in the seminiferous tubules, and predominantly in spermatids.

Consequently, taking these data into account the source of estrogens in rat testicular cells has been carefully re-examined, and an additional source of estrogens in purified pachytene spermatocytes (PS), round spermatids (RS), and spermatozoa *(65,66)* were discovered. This agrees with the data of Janulis et al. *(67)*. The amount of P450arom mRNA decreases according to the stage of germ cell maturation, being higher in youngercompared to mature rat germ cells. Conversely, aromatase activity is higher in spermatozoa than in PS or RS. All together the aromatase activity in germ cells represents about 60% of the whole testicular activity *(68,69)*. Similarly, in the bank vole, aromatase has been demonstrated in germ cells and its expression has been shown to be photoperiod-dependent, i.e., much more intense under longlight cycle conditions *(28)*.

Recent observations made in the seminiferous tubules of the stallion have shown a positive immunoreactive signal for aromatase in cytoplasm surrounding germ cells indicating the presence of aromatase in Sertoli cells *(37)*. Hess and Roser *(70)* who do not find any aromatase positive germ cells in horse testes suggest that it is may be related to antibodies binding characteristics and/or species differences, but in vitro studies on purified germ cells should be done.

In human Sertoli cells the presence of aromatase activity that is under control by germ cells *(71)* is described. Moreover, in fertile men, it has been shown recently that immature germ cells and ejaculated spermataozoa express a biolgically active aromatase *(72,73)*. These data confirm the observations of Hendry et al. *(11)*, showing that the concentration of estrogens in the rete testis fluid of men is higher than in the peripheral blood.

PHYSIOPATHOLOGICAL ROLE OF ESTROGENS AND FUTURE DEVELOPMENTS

In order to exert a biological role, testicular estrogens should interact with estrogen receptors (ERs), which in turn modulate the transcription of specific genes. Indeed, rat Sertoli and Leydig cells *(74)* contain ERs. However, until 1996, the only data on estrogen receptor concerned ER-α. However, with the discovery of a novel estrogen receptor designed ER-β, the localization of the ER has been re-examined, and it has been shown that the α-and β-forms are not always present in the same cells (or are present in different amounts)

within the male genital tract *(10,75)*. The distribution of the mRNAs of the two types (ER-α and ER-β) in the male rat gonad has been determined *(76)* and Saunders et al. *(77)* have shown the presence of ER-β in PS and spermatids. ER-β has been also immunolocalized in the Leydig cells and in the seminiferous tubules of the bank vole *(28)*. In primates, both ERs have been demonstrated but it is clear that Leydig cells mainly express ER-α whereas in seminiferous tubules, ER-β, and ER-α are present especially, in PS and RS *(69,73,78)*.

Mutembei et al. *(33)* have shown in the boar that ER-β is ubiquitously distributed in testicular cells including Leydig cells and is absent only in elongated spermatids. Conversely ER-α is very weakly expressed in Leydig cells and is more abundant in young germ cells (spermatogonia and PS). Therefore, species differences are obvious and the pig seems to have a unique distribution of ERs consitent with the large amount of estrogen produced in boar testes *(30)*.

Nevertheless a general statement can be made: ER-α is mainly localized in the Leydig cells, whereas ER-β is found in the seminiferous tubules and mainly in germ cells *(10)*.

The role of estrogens in the development and maintenance of the male gonadal functions is obvious: evidence has been provided concerning the somatic cells (Leydig and Sertoli cell), which are controlled by estrogens before puberty *(79,80)*. Recently, it has been shown that endogenous estrogens inhibit mouse fetal Leydig cell development *(81)*. It is also known that an estrogen-related protein control negatively the Leydig cell steroidogenesis in rat *(82)*. In pathological situations as Leydig cell tumors *(83,84)* and cryptorchidism *(85,86)*, an increase of aromatase activity is observed; indeed overexpression of aromatase in mice leads to the development of Leydig cell tumors *(87,88)*. Moreover, the estrogen sulfotransferase enzyme is expressed in Leydig cells of mice, rats and men *(89)*, and this enzyme may, therefore, play a role in male reproduction by regulating the amount of endogenous estrogen (by forming inactive sulphoconjugates).

In conclusion, the net biological effect of estrogen in the testis was thought to be mainly negative. Our knowledge of the role of estrogens in the male gonad has now been extensively revised in light of data obtained from knockout mice including the ER *(90)* and aromatase *(2)* gene deletions, and in aromatase-deficient men *(91)*. Even if numerous estrogen-targeted-genes remain to be defined, there is now a body of evidences in favor of a positive role for estrogens in male reproduction through genomic and rapid membrane effects *(92)*. Therefore age-related studies are necessary (especially in older animals) to fully understand the control of aromatase expression, and the role of estrogens (and environmental xenoestrogens) in Leydig cells and in male reproduction *(93–102)*.

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14 The Role of Estrogen Sulfotransferase in Leydig Cells

Wen-Chao Song, PhD

SUMMARY

Estrogen sulfotransferase (SULT1E1) is a cytosolic enzyme that catalyzes the sulfo-conjugation and inactivation of estrogens. It is expressed in the adult type of Leydig cells under the control of luteinizing hormone and androgen. Absence of Sult1e1 in the mouse leads to estrogen-induced Leydig cell hyperplasia and seminiferous tubule atrophy, abnormal accumulation of cholesterol ester and increased sensitivity to estrogen-induced steroidogenic lesion. The biochemical defects in Sult1e1-deficient Leydig cells is associated with the induction of scavenger receptor class B type-I (SR-BI), which leads to increased high density lipid-cholesterol uptake, and suppression of CYP17 expression and activity. It is concluded that SULT1E1 plays a physiological role in protecting Leydig cells and seminiferous tubules from excessive stimulation by the testis-produced estrogens and its inhibition by environmental chemicals, as recently demonstrated for derivatives of polychlorinated biphenyl, might have repercussions for human reproductive health.

Key Words: Androgen; cholesterol transport; estrogen; Leydig cells; steroidogenesis; sulfotransferase.

INTRODUCTION

Estrogen sulfotransferase (EST, SULT1E1) is a phase II drug-metabolizing enzyme which catalyzes the sulfo-conjugation of estrogens at the 3′-hydroxyl position of the phenolic ring (Fig. 1) *(1,2)*. Sulfated estrogens do not bind to the estrogen receptor (ER) and are therefore, hormonally inactive. Uncharacteristic for a drug-metabolizing enzyme, SULT1E1 shows high substrate specificity for endogenous estrogens and is catalytically active at physiological concentrations of the substrates *(1,3–5)*. Recent studies have revealed that a major site for SULT1E1 expression in mammalian species is the male reproductive tract *(6–9)*. In particular, Sult1e1 expression in the mouse testis was shown to be restricted to Leydig cells *(7,10)*, suggesting that the enzyme that play a physiological role in Leydig cell function and/or in maintaining testicular estrogen homeostasis. In this chapter, the biochemistry of SULT1E1 enzyme, its regulation in Leydig cells, and the physiological role of SULT1E1 as modeled by gene knockout mouse studies, will be reviewed.

BIOCHEMISTRY OF SULT1E1

SULT1E1 is a member of a family of enzymes known as cytosolic sulfotransferases *(1,2)*. These enzymes have molecular weights in the range of 30–34 kd in various mammalian species *(1,2)*, and catalyze the transfer of a sulfonate radical (SO_3^-) to a hydroxyl group in their respective substrates *(1,2)*. The SO_3^- group is provided by the activated sulfate donor, 3-phosphoadenosine-5-phosphosulfate, which acts as a cofactor for the sulfonation reaction *(1,2)*. Typical substrates for cytosolic sulfotransferases are small molecules of both endogenous and exogenous origins such as steroids, drugs, and xenobiotic chemicals *(1,2)*. The cytosolic sulfotransferases thus are distinct from another class of sulfotransferases which are membrane-bound and use macromolecules such as proteins, proteoglycanes, and glycosaminoglycans as their preferred substrates *(1,2)*.

Two members of the cytosolic sulfotransferase family have been characterized as having steroids as their natural substrates. SULT2A1 preferentially conjugates hydroxysteroids such as dehydroepiandrosterone and pregnenolone, whereas SULT1E1 has a substrate specificity for endogenous estrogens including estradiol, estrone, and estriol *(2,6,11,12)*. SULT1E1 also shows catalytic activity toward synthetic estrogens such as ethinyl estradiol and diethylstilbestrol, although the K_m values with these compounds are much higher than that

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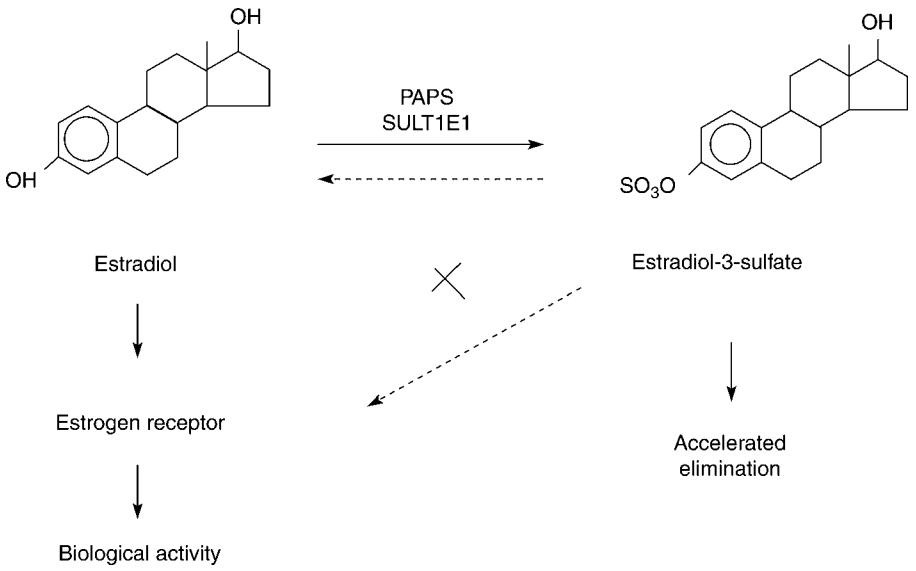


Fig. 1. Enzymatic reaction catalyzed by SULT1E1. The enzyme catalyzes the transfer of SO_3^- from 3-phosphoadenosine-5-phosphosulfate to the 3′-hydroxyl position of estrogens. Sulfo-conjugation of estrogens blocks their binding to the estrogen receptor and facilitates disposition of the hormone.

of natural estrogens *(4,5,13,14)*. Other sulfotransferases (SULT1A1 and SULT2A1) have also been reported to conjugate estrogens in in vitro experiments involving high concentrations of the substrates *(5,15)* but it is doubtful that these enzymes play a physiological role in regulating endogenous estrogen homeostasis in vivo.

Within the last decade, the cDNAs of SULT1E1 enzymes from a number of mammalian species have been cloned and the encoded proteins extensively characterized *(6,16–18)*. There is a high degree of homology at the amino acid level between SULT1E1 enzymes from different species. For example, the murine enzyme is 76 and 74% identical to the human and the guinea pig enzyme, respectively *(6)*. Some variations were observed in the degree of substrate specificity between SULT1E1 enzymes from different species *(14,19)*. For example, although the mouse EST shows little catalytic activity for hydroxysteroids *(19)*, the human enzyme is capable of sulfonating both pregnenolone and dehydroepiandrosterone *(4,19)*. Nevertheless, it is quite clear that estrogen is the natural and preferred substrate of all SULT1E1 enzymes that have been characterized *(4,6,16–19)*. The recombinant mouse and human SULT1E1 enzymes have also been crystallized and their three dimensional structures determined by X-ray crystallography *(20,21)*. The general scaffold of SULT1E1 enzymes shows a high degree of similarity to the structure of nucleotide kinases *(20,21)*.

EXPRESSION AND REGULATION OF SULT1E1 IN LEYDIG CELLS

Using a specific cDNA probe and a rabbit antimouse Sult1e1 antibody, the tissue expression pattern of murine Sult1e1 was investigated *(6)*. Surprisingly, in the initial study of normal mice, Sult1e1 mRNA was detected only in the testis *(6)*. In contrast, in the obese and diabetic *db/db* mice that harbor a mutation in the leptin receptor gene, Sult1e1 mRNA and protein were shown to be present at high levels in the liver as well as in the testis *(6)*. More recent studies have established that Sult1e1 is also expressed in the mouse epididymis and in mid to late stages of the mouse placenta *(8,22)*. Immunohistochemical staining revealed that within the mouse testis, Sult1e1 is exclusively localized to Leydig cells (Fig. 2). Although, no studies have yet specifically demonstrated Sult1e1 expression in Leydig cells of other mammals, the enzyme is known to be present in the testes of a number of mammalian species *(7,9,23,24)*.

Expression of Sult1e1 appears to be a unique feature of the adult type of Leydig cells and is under the control of the pituitary gland-secreted luteinizing hormone (LH). During the lifetime of a male animal, two populations of Leydig cells are produced in the testis *(25)*. A fetal type of Leydig cells develop prominently during embryogenesis. Testosterone secreted by these cells is essential for the development of a male phenotype. These cells regress gradually, after birth and are eventually replaced by the adult type of Leydig cells *(25)*. In the mouse, the adult

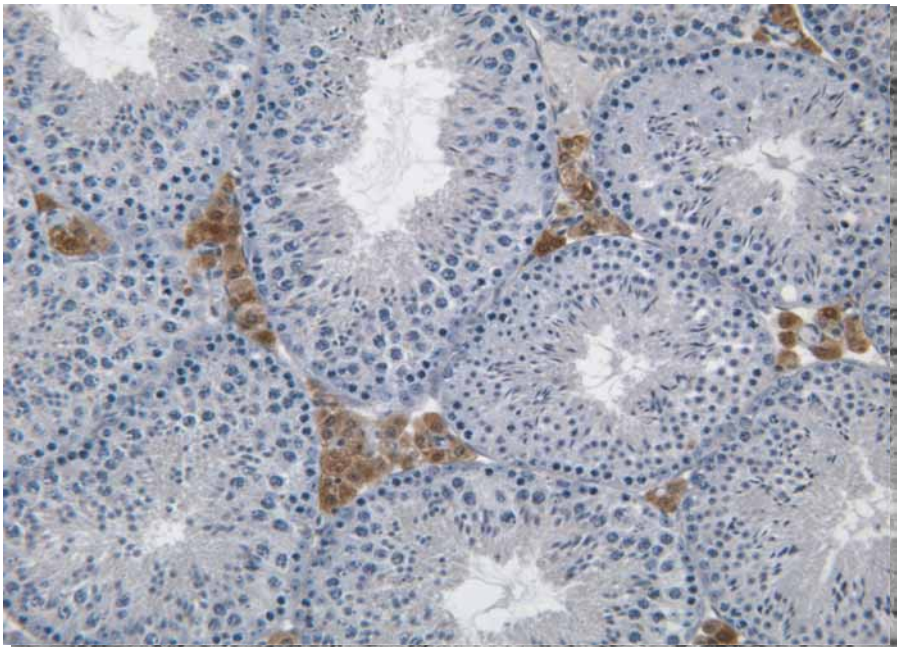


Fig. 2. Immunohistochemical staining of a mouse testis section shows that Sult1e1 is specifically expressed in Leydig cells. A polyclonal rabbit antimouse Sult1e1 was used as the primary antibody. (Please *see* color version of this figure in color insert following p. 180.)

type of Leydig cells first appear at about day 9 *postpartum* (p.p) and reach its maximum number around day 35 p.p. *(25,26)*. Studies have demonstrated that Sult1e1 is expressed in the testes of mature, 60-d-old mice but not in that of prepubertal, 5 or 17-d-old mice *(7)*. Furthermore, no Sult1e1 expression in the testes of either day 13.5 p.c. or day 15.5 p.c. mouse fetuses was detected *(7)*. As the adult-type mouse Leydig cells begin to differentiate from mesenchymal precursor cells during the second week of postnatal life *(26,27)*, one might expect the presence of a mixture of fetal and adult types of Leydig cells in the testis of a 17-d-old mouse. Thus, the fact that Sult1e1 was completely absent in the testes of 17-d-old mice *(7)* suggested that expression of Sult1e1 is not an inherent property of adult type Leydig cells but is likely to be regulated hormonally during sexual maturation.

Indeed, subsequent experiments determined that Leydig cell expression of Sult1e1 is critically regulated by pituitary hormone(s). It was shown that hypophysectomy completely abolished Sult1e1 expression in the mouse testis *(7)*. However, testicular Sult1e1 expression in hypophysectomized mice was readily restored after four consecutive daily injections of recombinant human chorionic gonadotropin (hCG) *(7)*. This result suggested that LH is necessary and sufficient for Leydig cell Sult1e1 expression. The regulatory effect of LH on Leydig cell Sult1e1 expression was further confirmed by experiments with primary-cultured mouse Leydig cells. Sult1e1 expression in cultured mouse Leydig cells was markedly elevated after the cells were stimulated with cAMP, a second message of LH action after receptor coupling *(28)*.

One of the downstream events stimulated by LH in Leydig cells is the activation of the steroidogenic pathway. Several lines of evidence supported the hypothesis that LH/cAMP act through androgen to regulate Sult1e1 expression in Leydig cells. First, aminoglutethimide, a steroidogenesis inhibitor, inhibited the cAMP-induced Sult1e1 expression in cultured Leydig cells *(28)*. Second, exogenously added androgen stimulated Sult1e1 expression in a dose-dependent manner, and this stimulation could be blocked by the androgen receptor antagonist, hydroxyflutamide *(28)*. Third, the cytokine IL-1β, which is known to inhibit Leydig cell steroidogenesis by negatively regulating CYP17 gene expression, blocked cAMP-induced Sult1e1 expression. Furthermore, the inhibitory effect of IL-1β on Sult1e1 expression could be reversed by the addition of exogenous androgen *(28)*. Finally, Sult1e1 is not expressed in the androgen receptor-insensitive *Tfm* mice, providing more direct evidence that receptor-dependent androgen action is required for Leydig cell Sult1e1 expression *(28)*.

The *Tfm* mouse model also allowed the direct vs the indirect effect of LH on *Sult1e1* gene expression to be differentiated. This mouse contains a natural mutation in the androgen receptor, and although there is impairment in Leydig cell development at puberty, total Leydig cell numbers in adult *Tfm* mice are more or less the same as

those in normal mice *(29)*. On the other hand, the lack of androgen activity abolishes the negative feedback mechanism of the hypothalamus–pituitary–testis axis, resulting in elevated serum LH level in adult *Tfm* mice *(29)*. The fact that CYP17, a sensitive marker gene for LH activity in Leydig cells, is still expressed prominently in the *Tfm* mouse testis suggests that the coupling of LH to its receptor is not blocked *(28)*. Thus, the lack of Sult1e1 expression in *Tfm* mice cannot be attributed to Leydig cell atrophy or lack of coupling between LH and its receptor. Additionally, the expression of both CYP17 and 3βHSD in *Tfm* mouse testis suggests that the lack of Sult1e1 expression is not a nonspecific phenomenon related to cryptorchidism *(28)*.

The stimulation of Leydig cell Sult1e1 expression by androgen appeared to be specific, as estrogen did not show the same effect *(28)*. Also, unlike in the androgen receptor-defective *Tfm* mice *(28)*, Sult1e1 was shown to be expressed abundantly in the Leydig cells of ER-α knockout mice *(28)*. The observation of androgen-regulated Sult1e1 expression in Leydig cells is consistent with results of other studies demonstrating a role of androgen in the regulation of hepatic and epididymal Sult1e1 expression *(8,18,30)*. Expression of Sult1e1in the mouse *(7)* and rat *(18)* liver is male specific, and in the case of the rat, it was shown to correlate with markers of androgen sensitivity in the liver *(18,30)*. Thus, Sult1e1 was expressed in the androgen-sensitive liver of mature adult rats but not in the androgen-insensitive liver of prepubertal or senile rats *(30)*. Furthermore, treatment with exogenous androgen, induced abnormal hepatic Sult1e1 expression in ovariectomized female rats *(30)*. Sult1e1 has also been shown to be expressed in the mouse epididymis in an androgen-dependent manner *(8)*. Together, these findings indicate that the stimulating effect of androgen on *Sult1e1* gene expression is not cell type specific, and that androgen can function both as an endocrine and an autocrine factor in this regard. The molecular mechanism by which androgen regulates Leydig cell Sult1e1 remains to be determined. However, there must be other factors, in addition to the androgen receptor, which participate in this process, as Sult1e1 is not expressed in all androgen target tissues or in fetal Leydig cells where androgen biosynthesis also takes place *(7)*.

ROLE OF SULT1E1 IN LEYDIG CELL FUNCTION AS REVEALED BY KNOCKOUT MOUSE STUDIES

The discrete and highly regulated expression of Sult1e1 in Leydig cells suggested that the enzyme play a physiological role in protecting Leydig cells from excessive estrogen stimulation and/or in maintaining estrogen homeostatsis in the testis. It is now well recognized that estrogens are both produced within the testis and required for normal testicular function *(31–36)*. Both ERs-α and -β and the estrogen biosynthetic enzyme, P450 aromatase, are expressed in various compartments of the testis including the Leydig cells *(37–39)*. Targeted deletion of ER-α in the mouse resulted in progressive damage of seminiferous tubules because of impairment of the absorptive function of the efferent ductules in the proximal epididymis, ultimately producing a male infertility phenotype *(31,33)*. Likewise, disruption of the gene encoding P450 aromatase caused an age-dependent decline in spermatogenesis, which eventually rendered male P450 aromatase knockout mice sterile *(35,36)*. These phenotypes of mice lacking estrogen signaling suggest that estrogen exerts a key regulatory role in male reproduction through an ER-mediated mechanism.

In addition to serving the local estrogen needs in the male reproductive system, the estrogen biosynthetic pathway in the testis also contribute significantly to the systemic estrogen pool from which estrogens required by extragonadal tissues like the bone is derived *(40,41)*. Indeed, it was determined that the concentration of estrogen in the human spermatic vein is 50 times higher than that in the systemic circulation *(42)*, and it is well appreciated that the testis is a significant source for peripheral estrogens in male animals and man *(40–42)*.

Although a physiological role for estrogens in normal testicular function is firmly established, unregulated estrogen activity within the testis also be detrimental. It is well documented that application of exogenous estrogens to male animals inhibited their spermatogenesis *(43)*, both through a direct effect on the testis and by reducing androgen production through the negative feedback mechanism of the hypothalamus-pituitary-testis axis *(44,45)*. In some susceptible strains, exogenous estrogens are known to cause Leydig cell tumors *(46)*. Transgenic expression of P450 aromatase has also been shown to predispose mice to Leydig cell tumor induction *(47)*. At a biochemical level, estrogens are known to inhibit steroidogenesis of Leydig cells through selective inhibition of the 17,20-lyase activity of CYP17, a key enzyme in androgen biosynthesis that catalyzes the conversion of progesterone to androstenedione or pregnenolone to dehydroepiandrosterone *(48–51)*. Given its high substrate specificity toward endogenous estrogens, it be expected that Leydig cell Sult1e1 functions as a local estrogen modulator within

the testis to protect Leydig cells and other cell types from excessive estrogen stimulation.

The possible role of Sult1e1 in vivo has been addressed by gene knockout studies *(8,10,22,52)*. Murine Sult1e1 is a single copy gene localized on chromosome 5 (http://www.informatics.jax.org/). Genomic clones containing the mouse *Sult1e1* gene were isolated and the exon/intron organization of the gene characterized *(10)*. It was found that the mouse *Sult1e1* gene consists of 8 exons, spanning a region of approx 15–20 kb *(10)*. A gene-targeting vector was constructed, using a 13 kb genomic fragment, which contained exons 2 to 7 *(10)*. The targeting strategy was to insert a *neo* gene cassette into exon 3, resulting in the disruption of the normal coding frame of the *Sult1e1* gene and producing a null mutation of the protein *(10)*. The targeted gene allele was differentiated in Southern blot analysis by using *XbaI*-digested genomic DNAs *(10)*. In this screening strategy, the wild-type (WT) *Sult1e1* gene allele showed a 6kb band while the mutated gene allele a 7 kb band because of *neo* gene insertion into exon 3 *(10)*. Successful disruption of the *Sult1e1* gene in mice was confirmed by the absence of mRNA and protein in the mutant mice *(10)*.

Characterization of knockout male mice aged 3–6 mo revealed no apparent functional abnormalities in their reproductive system. There was no statistical difference in serum testosterone or free estradiol levels between wild-type and knockout males *(10)*. In an initial mating experiment using five breeding pairs of wild-type or knockout mice, the knockout pairs were found to produce fewer litters and had smaller litter size *(10)*. However, subsequent crossbreeding experiments in which wild-type males were paired with knockout females or wild-type females were paired with knockout males revealed that the reduced fertility in the mutant mice originated from placental abnormality during mid-gestation *(10,22)*.

Further studies uncovered some age-dependent Leydig cell abnormalities in *Sult1e1* gene knockout mice. Although no numerical or morphological difference was observed between 2-mo-old wild-type and knockout mouse Leydig cells, Leydig cell hyperplasia and hypertrophy was commonly observed in the testes of 12 mo or older Sult1e1 knockout mice *(10)*. Compared with wild-type cells, the cytoplasm of the knockout Leydig cells stained less uniformly with eosin on HE staining and contained vacuoles resembling those found in adipocytes (Fig. 3). Interestingly, hyperplasia occurred more frequently (although, not exclusively) in the peripheral space under the testicular capsule, suggesting that mesenchymal precursor cells located in this area were the targets of estrogen-stimulated proliferation *(10)*. The second abnormality found in Leydig cells of testes of the knockout mouse was the presence of numerous "giant cells" that stained yellow on HE staining *(10)* and strongly positive with periodic acid-Schiff (PAS) staining. These cells contained multiple nuclei and were detected in the hyperplastic lesions found in the peripheral space as well as in Leydig cell clusters located at inner tubular junctions *(10)*. Finally, disrupted seminiferous tubule structures were observed in the majority of testis sections of 12-mo and older knockout mice (Fig. 3). The abnormal tubules were filled with vacuoles and had reduced germinal cell layers (Fig. 3). Notably, such abnormal seminiferous tubules were usually localized adjacent to hypertrophic and hyperplastic Leydig cell lesions (Fig. 3). These findings suggest that Sult1e1 acts in an intracrine manner to suppress estrogen activity within Leydig cells as well as works in a paracrine fashion to reduce estrogen levels in the local microenvironment in the testis. An alternative explanation for the seminiferous tubule phenotype is that the hypertrophic/hyperplastic Leydig cells release other nonsteroidal paracrine factors *(25)* that could negatively impact the local microenvironment, ultimately leading to the development of the observed seminiferous tubule disruption.

Interestingly, the structural abnormalities in Leydig cells of aged knockout mice could be recapitulated in younger (2–3 mo old) knockout mice by exogenous estrogen treatment *(10)*. Treatment with exogenous estradiol did not cause appreciable Leydig cell damage in the wild-type mice, but it resulted in severe Leydig cell hypertrophy/hyperplasia and seminiferous tubule damage which were similar to that observed in untreated 12-mo-old mice (Fig. 3). Thus, exogenous estrogen accelerated the development of structural lesions in the knockout mouse testis. This finding supports the conclusion that increased estrogen activity consequential to Sult1e1 deficiency was the underlying cause of the observed testicular phenotype in aged knockout mice.

The hypertrophic Leydig cells in aged knockout mice and in estrogen-challenged young knockout mice resembled adipocytes in appearance. This led to the speculation that the hypertrophy might be caused by abnormal lipid accumulation in these cells. Indeed, staining with Oil-Red O of frozen sections of wild-type and knockout mice revealed a dramatic accumulation of neutral lipids in the mutant mouse Leydig cells *(52)*. Subsequent thin-layer chromatography (TLC) and ELISA analysis determined that the abnormally accumulated lipid in the mutant mouse Leydig cells was primarily cholesterol ester in nature *(52)*.

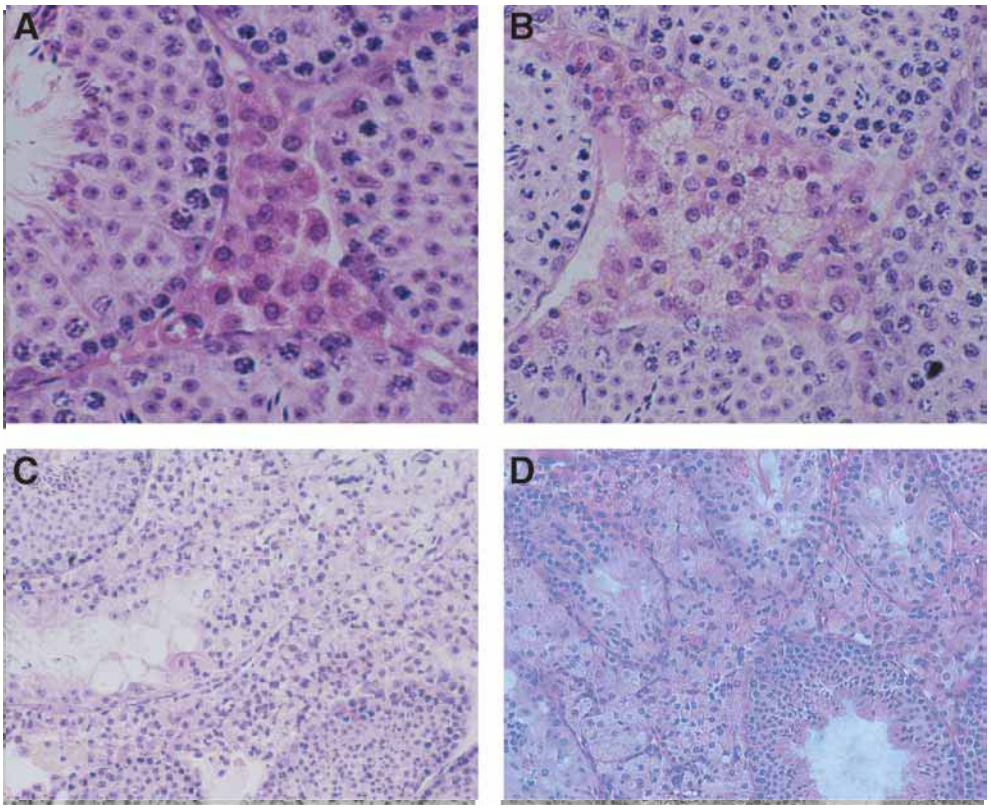


Fig. 3. Leydig cell and seminiferous tubule abnormalities in Sult1e1 knockout mice. Compared with normal Leydig cells in wild-type mice **(A)**, Leydig cells in aged Sult1e1 knockout mice appear hypertrophic and hyperplastic **(B,C)**. There is also seminiferous tubule atrophy **(C)**. Similar changes in Leydig cells and seminiferous tubules could be produced in young Sult1e1 knockout mice by exogenous estrogen challenge **(D)**. (Please *see* color version of this figure in color insert following p. 180.)

Several lines of evidence suggested that increased cholesterol ester, accumulation in the mutant mouse Leydig cells resulted from enhanced uptake of cholesterol ester rather than decreased utilization of cholesterol as a precursor in the steroidogenic pathway. First, by Northern blot, Western blot and immunofluorescence studies, it was demonstrated that SR-BI, a major high density lipid receptor which mediates the selective uptake of cholesterol ester in liver and steroidogenic cells, was significantly upregulated in Sult1e1 knockout mouse Leydig cells *(52)*. This finding was consistent with the observation that estrogen administration induced SR-BI expression in the rat adrenal gland and ovary *(53)*. Second, through the use of a fluorescent high-density lipid-cholesterol ester, it was shown that primarily cultured Sult1e1 knockout mouse Leydig cells had rapid cholesterol ester uptake, whereas, the same phenomenon was not observed with cultured Leydig cells from wild-type mice *(52)*. Finally, no significant change in StAR expression was detected in the testis of aged or estrogen-treated Sult1e1 knockout mice *(52)*. StAR is a protein that facilitates cholesterol transport across the mitochondrial membrane and is essential for initiating the steroidogenic cascade *(54)*. Mutations of the StAR gene in human and mice caused lipoid adrenal hyperplasia, a condition characterized by abnormal cholesterol buildup in adrenal cortical cells because of the lack of cholesterol metabolism, *(55,56)*. Thus, the phenotype of abnormal cholesterol ester accumulation in Sult1e1 knockout mouse Leydig cells arose by a mechanism which is different from that which operates in lipoid adrenal hyperplasia *(56)*. It remains to be determined whether other mechanisms, such as altered intracellular cholesterol biosynthesis and/or cholesterol ester hydrolysis, abnormal expression or function of sterol carrier protein 2 and peripheral benzodiazepine receptor *(57,58)*, might also have played a role in the pathogenesis of the mutant mouse Leydig cell phenotype.

In addition to the disturbance in cholesterol homeostasis, Sult1e1 knockout mouse Leydig cells were also shown to be susceptible to developing estrogen-induced steroidogenic lesion. In a comparative study, in which the steroidogenic capacity of Leydig cells from estrogen-treated 3-mo-old wild-type and Sult1e1 knockout

mice was evaluated, Sult1e1-deficient Leydig cells in culture produced 50–70% less testosterone than the wild-type cells *(52)*. It is unlikely that this steroidogenic defect was caused by abnormal cholesterol mobilization as a result of an addition of 22R-hydroxycholesterol, an exogenous substrate that can bypass the need of a StAR-mediated cholesterol transport mechanism, failed to correct the defect *(52)*. The conclusion that abnormal cholesterol transport is not responsible for the steroidogenic lesion is also supported by the finding that Normally StAR is expressed in these cells *(52)*.

Further experiments established that the steroidogenic lesion was caused by a reduction in *CYP*17 activity *(52)*. It was shown that androstenedione but not progesterone supplementation reversed the deficiency in testosterone production by estrogen-treated Sult1e1 knockout mouse Leydig cells *(52)*. Furthermore, investigation by Northern blot and Western blot analysis of the downstream steroidogenic enzyme system revealed a significant reduction in *CYP*17 expression but no changes in the expression levels of P450scc and 3β-HSD were observed *(52)*. Concomitant with reduced testosterone production, progesterone level in cell culture medium of Sult1e1 knockout mouse Leydig cells was abnormally elevated *(52)*, further supporting the conclusion that *CYP*17 activity was reduced.

The finding of estrogen-induced *CYP*17 inhibition in the Sult1e1-deficient mouse Leydig cells is consistent with previous studies that demonstrated similar effects of exogenous estrogen on Leydig cells. For example, the synthetic estrogen DES was found to inhibit Leydig cell *CYP*17 activity in Balb/c mice *(59)*. In a hypophysectomized rat model, both DES and 17β-estradiol were found to inhibit Leydig cell *CYP*17 expression and activity *(51,60)*. In the latter setting, Leydig cells from hypophysectomized rats have partially or completely inhibited Sult1e1 expression as Sult1e1 expression in the mouse Leydig cells was totally abolished by hypophysectomy *(28)*. Additionally, the estrogen sensitivity of wild-type Leydig cells in these settings was likely related to the fact that DES is a relatively poor substrate of Sult1e1 *(14)*. Thus, lack of Sult1e1-dependent modulation of estrogen activity be responsible as well for the observed estrogen sensitivity in these other studies *(50,51,60)*.

CONCLUDING REMARKS

The requirement of estrogen in male as well as female reproduction in mammalian species is now firmly established. The discrete and highly regulated expression in Leydig cells of Sult1e1, an estrogen-specific metabolic enzyme, suggests that local estrogen activity must be carefully controlled to avoid excessive estrogen stimulation. This proposition is fully supported by genetic studies of mice which have a targeted mutation of the *Sult1e1* gene. In this context, it is of considerable interest to note that recent enzymatic and crystallographic studies have identified human SULT1E1 as a target of potent inhibition by hydroxylated polychlorinated biphenyls *(61,62)*, a class of environmental chemicals that are suspected as potential endocrine disrupters in human and animal species *(63–69)*. The phenotype of Sult1e1 knockout mice suggests that inhibition of SULT1E1 activity by environmental agents such as hydroxylated polychlorinated biphenyls produce Leydig cell lesions, as well as cause abnormality in other estrogen target tissues where SULT1E1 is expressed. Thus, by inhibiting estrogen transformation enzymes such as SULT1E1, an otherwise nonestrogenic chemical become highly estrogenic in vivo by potentiating endogenous estrogen activity. This constitute a previously unrecognized mechanism of action for endocrine disrupting chemicals, generally, which are thought to act directly on steroid hormone receptors as estrogen mimics or antiandrogens *(63–69)*. Further studies on the role and regulation of Sult1e1 in Leydig cells and other estrogen target tissues are of significant relevance to human reproductive health.

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15 Endocrinology of Leydig Cells in Nonmammalian Vertebrates

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SUMMARY

A review of testicular structure and function in nonmammalian vertebrates indicates that a comparative approach can expand our view of a subject through the dimension of evolutionary time; provide a conceptual framework for identifying unifying principles; separate fundamentally conserved features from species-specific specializations; and help to identify important unanswered questions. Comparative studies to date support the following generalizations:

1. Leydig cells are relative newcomers to the cellular repertory of the vertebrate testis.
2. Leydig cells evolved as a source of circulating steroids, eventually supplanting Sertoli cells as the primary steroidogenic element of the testis.
3. Male germ cell production and development requires an androgen-rich environment in all vertebrate classes, but the contribution of Leydig cells vs Sertoli cells to the intratesticular steroidogenic *mileu* varies as a function of phyletic position, developmental age, time of year, and physiological status.
4. From fish to mammals, seminiferous tubules progressively displace spermatocysts as the primary organizational unit and germinal compartment of the testis.
5. The evolutionary origin of Leydig cells and a trend toward increased testicular structural and functional complexity exactly parallel the evolution of the seminiferous tubule and concomitant acquisition of a second testicular compartment, the interstium. The evolution of the testes with higher organizational complexity and more diverse cellular repertory, including Leydig cells, is just one aspect of a multitude of reproductive adaptations that accompany animal evolution *per se*. Among vertebrates, species with a relatively simple testicular organization and fewer testicular cell types are advantageous for addressing research questions.

Key Words: Leydig cell; Sertoli cell; spermatogenesis; evolution; steroidogenesis; nuclear receptors.

HISTORY AND RATIONALE OF COMPARATIVE STUDIES

Historically, pathbreaking advances in formulating "the cell theory," and our understanding of germ cells, fertilization, embryogenesis, spermatogenesis, and andrology, were made by investigators who used a comparative microscopical approach to observe the testes and semen of a wide variety of animals and man (for review *see* refs. *1* and *2*). Especially relevant here is the detailed account of the male sex organs published by Franz von Leydig in 1850 *(3)*. In this treatise, von Leydig credited the comparative approach with his ability to recognize the special features of the interstitial cells surrounding the blood vessels and seminiferous tubules of the testis, and their relationship with active spermatogenesis. Although the wide choice of animal models in these early studies was driven mainly by practical considerations, a purposeful phylogenetic analysis of contemporary animal species is an approach that can expand our view of a subject through the dimension of evolutionary time. According to the eminent geneticist Theodosius Dobzhansky, "nothing in biology makes sense except in the light of evolution" *(4)*.

The main benefit of an evolutionary perspective is that it provides a conceptual framework for recognizing unifying principles, separating fundamental conserved features from relatively trivial specializations, and identifying important unanswered questions. Ultimately, it is this knowledge that provides a sound scientific rationale for extrapolating animal studies to the human

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condition. Additionally, broadening our information base from a mere handful of organisms to representatives of the remaining more than 42,000 vertebrate species *(5)* can be expected to reveal a few with advantageous features that can be used to address new research problems. In the past, the course and causes of evolution were mainly the domain of paleontologists, anatomists, and embryologists, but the emergence of comparative functional genomics makes it possible to measure similarities and differences among organisms with a fair degree of accuracy. The account that follows is intended to illuminate our understanding of the origin and changing role of Leydig cells during evolution. A comprehensive literature review and original citations pertaining to the subject can be obtained from earlier works *(1,6–8)*.

PHYLOGENETIC DEVELOPMENT OF LEYDIG CELLS AND THE ORGANIZATION OF THE TESTIS

As defined by the rodent prototype, our view of the structure and function of Leydig cells does not apply to the majority of vertebrates. Comparative studies instead support the following generalizations:

1. Leydig cells are relative newcomers to the cellular repertory of the vertebrate testis.
2. Leydig cells evolved as a source of circulating steroids and supplanted Sertoli cells as the primary steroidogenic element of the testis.
3. Male germ cell production and development requires an androgen-rich environment in all vertebrate classes, but the contribution of Leydig cells vs Sertoli cells to the steroidogenic mileu varies as a function of phyletic position, developmental age, time of year, and physiological status.
4. Seminiferous tubules progressively displace spermatocysts as the primary organizational unit of the testis, a process that culminates in the evolution of a permanent seminiferous epithelium.
5. The evolutionary origin of Leydig cells and a trend toward increased structural and functional complexity exactly parallel the evolution of the seminiferous tubule and concomitant formation of a second testicular compartment, the interstitium.

Primitive Fishes

The jawless fishes (Agnathans: lampreys, hagfish) lack seminiferous tubules and excurrent ducts. Instead, the primary organizational unit of the testis is the spermatocyst, a discrete follicle-like structure bounded by an acellular basal lamina. Each spermatocyst is made up of a clone of syncitially connected germ cells and a second clonal population of Sertoli cells, which are stage-synchronized in development *(9)*. In the protochordate amphioxus *Branchiostoma,* which is thought to resemble an ancestor of the vertebrate line, a bilateral pair of spermatocysts present in each of the central body segments collectively form the testis, but in the cyclostome fishes, multiple spermatocysts are loosely organized in a single medial testis. Because of the absence of seminiferous tubules, no true interstitum is present. The "interstitial" cells described in lampreys and hagfish resemble fibroblast-like mesenchymal precursors of Leydig cells, or could be Sertoli cells, which have, by contrast, relatively well-developed misidentified steroidogenic organelles *(9–13)*.

Cartilaginous Fishes

The presence or absence of Leydig cells at the next phyletic level (Elasmobranchs: sharks, skates, rays) is a matter of debate. What holds true for all species examined thus far, is that spermatocysts are present but embedded in a connective tissue matrix and attached to spermatic cords by short stalks. These cords do not become patent until the end of spermatogenesis, whereby the germ cells exit the testis. Although there are no definitive seminiferous tubules or interstitium, the empty spermatic cords and stromal elements can be viewed as presumptive tubules and interstitium, respectively. Electron microscopic studies in this laboratory observed undifferentiated Leydig-like cells in the interstices of spermatocysts in the testis of the spiny dogfish *(Squalus acanthias) (14)*. These cells were small, few in number, and mesenchymal in appearance, but they had a sparse agranular reticulum, tubulovesicular mitochondria, and lipid droplets, organelles that are typical of steroid producing cells. Because steroidogenic organelles are more highly developed in the greatly expanded Sertoli cells of the same species *(15)*, these authors postulated that the Leydig-like cells of dogfish sharks are the evolutionary forerunners of true vertebrate Leydig cells *(16)*. More recently, Prisco and coworkers, also using electron microscopy, reported cells with many Leydig cell features in the interstices of spermatocysts in the testis of the mature ray *Torpedo marmorata (17)*. Although small and sparse, like those described in the dogfish, these cells were rounded in appearance, with more abundant cytoplasm, smooth reticulum, lipid droplets, and mitochondria with tubular cristae. Interestingly, after spermiation, the cytological features characteristic of steroidogenesis were reduced, and the cells regressed to a fibroblast-like

shape, suggesting some functional interaction with developing germ cells.

Bony Fish

Fully mature epithelioid Leydig cells with abundant steroidogenic organelles are first seen in phylogeny at the level of bony fish. Teleosts also have seminiferous tubules (alternatively termed lobules), but they enclose spermatocysts *(18–20)*. Two main patterns of tubule organization exist, i.e., the restricted and unrestricted forms. The restricted form is limited to Antheriniformes and is typified by the killifish *Fundulus*. Tubules extend from the efferent ducts, or a centrally located collecting duct, into the body of the testis and contain spermatocysts arranged in descending maturational order; however, the boundary wall of the tubules is incomplete at their distal ends where the germinal zone (GZ) is located and cysts with primary spermatogonia and stem cells are embedded directly in a connective tissue matrix *(18,21)*. Leydig cells develop seasonally in the interstitium but only in regions where tubules contain mature or spermiated cysts. The spatial and temporal juxtaposition of Leydig cell development with the release or impending release of mature spermatozoa suggests their involvement in preparing the excurrent ducts for maintenance and transport of sperm, or in regulating extratesticular processes (e.g., sex behavior). At least one mammal, the squirrel *Citellus lateralis,* has clusters of Leydig cells surrounding the efferent ducts *(22)*.

The second teleostean pattern (unrestricted) is limited to Salmoniformes, Cyprinoformes, and Perciformes and is typified by that of the mullet *Mugil*. There is a branching network of tubules with stem cells of the germ cell and Sertoli cell lineages located at regular intervals along their length rather than in a defined GZ at one end. As new cysts are formed adjacent to the basement membrane, maturing spermatocysts are displaced progressively toward the lumen. Following rupture of fully mature cysts, sperm bundles and Sertoli cells are released into the lumen. This pattern resembles the base-to-lumen progression of germ cell generations in the seminiferous epithelium of higher vertebrates and, like these groups, Leydig cells are scattered among the tubular interstices *(23)*.

Regardless of tubule organization, and the location of fully differentiated Leydig cells, Sertoli cells are a prominent feature of the teleostean testis. Seasonal and stage-related variations in size, shape, the number and development of steroidogenic organelles, lipid droplets, cholesterol, and histochemical staining for 3β-(HSD) hydroxysteroid dehydrogenase correlate well with germ cell development in spermatocysts of some species, but stage-related or seasonal cyclicity is more subtle in others *(7,9,19,20,24)*.

Amphibians

Amphibians sit at the branch point of Leydig cell evolution. Urodeles (newts and salamanders) resemble teleosts with a restricted pattern of tubule development. Hypertrophy and differentiation of Leydig cells is keyed to spermiation. The "zonal" testis of the salamander *Necturus maculosus* has been studied in some detail *(25–30)*. In this animal, cysts with spermatogonia are located in tubular regions proximal to the GZ on the medial aspect of the testis. At the onset of seasonal recrudescence, immature cysts are displaced peripherally by proliferation of stem cells and formation of new spermatocysts. The distally located cysts now resume maturation and become the source of the current season's spermatozoa. This maturational sequence begins first at the posterior end and progresses anteriorly. Therefore, for a brief period in the seasonal cycle, subcapsular tissues in anterior regions contain cysts with mature spermatozoa, central regions have spermiating or empty lobules, and posterior regions have fully regressed or degenerating lobules. Although, Leydig-like cells are interspersed among the seminiferous tubules in regions containing immature or maturing spermatocysts, they are indistinguishable from fibroblasts except at the electron microscopic level *(28–30)*. However, adjacent to cysts undergoing spermiation, Leydig cells begin to hypertrophy and to acquire abundant steroidogenic organelles. These events are so tightly coupled that, in a given cyst, Leydig cells adjacent to spermiated regions are hypertrophied, whereas those adjacent to regions with spermatozoa in place are still in the undifferentiated state.

In contrast to urodeles, anuran amphibians (frogs and toads) more closely resemble teleosts with the unrestricted pattern of tubular organization and have fully differentiated Leydig cells diffusely located in the tubular interstices *(19,20,31–33)*. Anurans also display a feature that suggests an initial step in the evolution of a permanent seminiferous epithelium. That is, although spermatocysts are housed within a tubular compartment, Sertoli cells of mature cysts attach to the basement membrane of the tubule. Cysts rupture relatively early in the maturational progression (late spermatocyte stage). Although Sertoli cells remain attached, the germinal clones still embedded in depressions and laminae of Sertoli cells are now relatively open to the tubular lumen while completing development.

At spermiation, Sertoli cells of spent cysts detach from the tubule surface and exit the testis or form aggregates of degenerating remnants in the lumen. These disappear upon formation of the next generation of germinal cysts *(34)*.

In addition to steroidogenically competent Leydig cells, both urodele and anuran amphibians have Sertoli cells that display the cytological features of steroidogenesis, including positive staining for 3β-HSD and cholesterol-rich lipid droplets, organelles that have been correlated with the annual onset and regression of spermatogenesis, and are maximal toward the end of the germ cell development *(7,19,31–33,35,36)*.

Reptiles, Birds, and Mammals

All higher vertebrates have definitive seminiferous tubules and interstitial Leydig cells with highly developed steroidogenic organelles *(2,19,33,37)*. In these groups, a permanent seminiferous epithelium replaces the cystic mode of spermatogenesis of anamniote vertebrates. The homolog of the anamniote spermatocyst is the syncitially connected germ cell clone together with a cohort of Sertoli cells which it is associated; however, these germinal clones are not closed or spherical in shape but have a more flattened form with an extremely irregular three-dimensional outline. They appear to lie in "open communion" and share their Sertoli cell associations with other clonal generations within the seminiferous epithelium *(2)*. Unlike the situation in vertebrates with "zonal" testes, stage-related variations in the distribution or cytological features of Leydig cells of most reptiles, birds, and mammals are not readily discernible; however, the seminiferous epithelium of seasonal breeders tends to be more synchronized throughout the testis, a feature that facilitates analysis of Leydig cells and Sertoli cells in relation to a particular stage of spermatogenic development *(38–40)*.

STEROID SYNTHESIS AND SECRETION

The testis of all vertebrates, regardless of the presence or differentiated state of Leydig cells, has steroidogenic and steroid secretory capacity (for review *see* ref. *1*). Whereas early studies relied on tissue extracts and conventional biochemical methods or bioassays for steroid identification, the availability of high specific activity radiolabeled substrates enabled researchers to definitively identify steroidogenic pathways. Subsequently, commercially available immunoassays were applied to peripheral plasma, testicular tissues, or spent media. As shown in Table 1, the last decade is notable for the success of homology-cloning approaches to isolate complementary DNAs (cDNAs) encoding key steroidogenic enzymes in many different vertebrate species. In addition, cDNA fragments encoding the mitochrondrial steroidogenic acute regulatory (StAR) protein have been cloned from chicken, frog, and zebrafish, indicating that mechanisms regulating cholesterol shuttling in steroidogenic organelles are also conserved *(41)*. Comparison between cytochrome P450 (*CYP* gene) sequences in the human and pufferfish *(Fugu)* genomes reveals that the defining characteristics of vertebrate P450s have not changed much in the 420 million years since the divergence of fish and tetrapods, and only one new family *(CYP39)* is seen in mammals *(42)*. *CYP19*, the gene encoding P450 aromatase, the key enzyme in estrogen biosynthesis, is estimated to have diverged from the main *CYP* line about 800×10^6 yr before present *(43)*. In contrast to humans, however, the most teleost fish have two *CYP19* genes, termed –A and –B, which are believed to result from a whole genome duplication event in the ancient fish lineage *(44,45,176)*. The presence of only one *CYP19* gene in eels *(46)* most likely reflects secondary loss of one of the duplicated genes, because eels have many other teleostean duplicates (*see* "Nuclear Steroid Receptors"). In contrast to the three *CYP19* genes in pigs, which share 87–93% sequence identity *(47,48)*, the B- and A-paralogs of fish are only about 61% identical *(45)*, indicating a much longer evolutionary history as separate genes.

Application of sequence information and reagents to testis research in nonmammalian models has been somewhat slow; however, the proliferation of genome projects has facilitated bioinformatics approaches to search for orthologous genes in key invertebrate species. To date, putative *CYP11, CYP19, CYP20*, and *CYP 21* sequences have been identified in genomic and EST databases in the protochordate amphioxus, and *CYP11* and *CYP20* sequences have been found in the tunicates *(Ciona intestinalis* and *C. savignyi) (49)*. Additionally, an ortholog of 17β-hydroxysteroid dehydrogenase (-HSD) has been assembled from *Ciona* ESTs *(50)*. Although definitive proof of functional protein remains to be established for most reported sequences in invertebrates, high levels of aromatase enzyme have been identified in testis-containing segments of amphioxous *(25)*, and antipeptide antibodies immunolocalized 17β-HSD protein to interstitial cells of *Ciona* gonad *(50)*. Taken together, the evidence supports the view that steroidogenesis is a phylogenetically ancient process that originated in primitive life forms well in advance of the first appearance of vertebrate Leydig cells.

Table 1
Cloning of Steroidogenic Enzymes in Nonmammalian Vertebrates

Common name	*Scientific name*	*GenBank no.*	*mRNA expression in testis*
P450 cyp19 (Aromatase)			
Chicken	*Gallus gallus*	J04047	–
Zebra finch	*Taeniopygia guttata*	S75898	ND *(165)*
Red-eared slider turtle	*Trachemys scripta*	AF178949	–
American alligator	*Alligator mississippiensis*	AAK31803	–
African clawed frog	*Xenopus laevis*	BAA90529	Y *(166)*
Noboribetsi salamander	*Hynobius retardatus*	AAB204518	Y *(167)*
Iberian ribbed newt	*Pleurodeles waltl*	AY135485	Y *(168)*
Wrinkled frog	*Rana rugosa*	AB178482	–
Tilapia mossambica	*Oreochromis mossambicus*	(A) AF1358501	–
		(B) AF1358500	–
Nile tilapia	*Oreochromis niloticus*	(A) AF472621	ND *(169)*
		(B) AF306786	Y *(170)*
Medaka	*Orizyas latipes*	(A) D82968	ND *(171)*
		(B) AY319970	–
Red-spotted grouper	*Epinephelus akaara*	(A) AY547354	–
		(B) AY547353	–
Orange-spotted grouper	*Epinephelus coiodes*	(A) AY510711	Y *(172)*
		(B) AY510712	–
Sea bass	*Dicentrarchus labrax*	(A) AJ311177	Y *(173)*
		(B) AY138522	Y *(174)*
Wrasse	*Halichoeres tenuispiniss*	(A) AY489061	Y *(175)*
		(B) AY489060	Y *(175)*
Rainbow trout	*Oncorhynchus mykiss*	(BI) AJ311937	–
		(BII) AJ311938	–
Goldfish	*Carassius auratus*	(A) AF020704	–
		(B) AB009335	–
Zebrafish	*Danio rerio*	(A) AF004521	Y *(49,93)*
		(B) AF120031	Y *(49,93)*
Atlantic killifish	*Fundulus heteroclitus*	(A) AY428665	ND *(161)*
		(B) AY428666	ND *(161)*
Goby	*Trimma okinawae*	(A) Not reported	LC *(177)*
		(B) Not reported	Y *(177)*
Atlantic salmon			
Japanese flounder	*Paralichthys olivaceus*	(A) AB017182	Y *(178)*
Southern flounder	*Paralichthys lethostigma*	(A) AY863197	Y *(179)*
Atlantic halibut	*Hippoglossus hippoglossus*	(A) AJ410171	–
Swamp eel	*Monopterus albus*	(A) AY583785	–
European eel	*Anguilla anguilla*		Y *(45)*
Japanese eel	*Anguilla japonica*	(A) AY540622	–
Gilthead seabream	*Sparus aurata*	(A) AF399824	–
Red seabream	*Pagrus major*	(A) AB051290	–
Porgy	*Acanthopagrus schlegelii*	(A) AY273211	Y *(180)*
Channel catfish	*Ictalurus punctatus*	(A) S75715	Y *(181)*
		(B) AF120031	Y *(194)*
Southern catfish	*Silurus meridionalis*	(B) AY325907	–
Pejerrey	*Odontesthes bonariensis*	(B) AY380061	–
Fathead minnow	*Pimephales promelas*	(B) AJ277866	Y *(182)*
Atlantic stingray	*Dasyatis sabina*	AF097513	–
Spiny dogfish	*Squalus acanthias*	DQ223101	–

(Continued)

Table 1 *(Continued)*

Common name	*Scientific name*	*GenBank no.*	*mRNA expression in testis*
Little skate	*Leucoraja erinacea*	DQ223102	Y *(183)*
Amphioxus	*Branchiostoma floridae*	DQ165086	–
		DQ085624	–
P450 11A1 (Side Chain Cleavage)			
Chicken	*Gallus gallus*	D49803	–
Rainbow trout	*Oncorhynchus mykiss*	S57305	–
Japanese eel	*Anguilla japonica*	AY654741	–
Channel catfish	*Ictalurus punctatus*	AF063836	Y *(184)*
Southern stingray	*Dasyatis Americana*	U63299	–
P450c17 (17α-hydroxylase/17, 20-lyase)			
Chicken	*Gallus gallus*	M21406	–
African clawed frog	*Xenopus laevis*	AF325435	–
Rainbow trout	*Oncorhynchus mykiss*	X65800	–
Zebrafish	*Danio rerio*	AY281362	Y *(185)*
Fat head minnow	*Pimephales promelas*	AJ277867	Y *(186)*
Japanese eel	*Anguilla japonica*	AY498619	–
Rice field eel	*Monopterus albus*	AY224681	Y *(187)*
Channel catfish	*Ictalurus punctatus*	AF063837	Y *(184)*
Spiny dogfish	*Squalus acanthias*	S77384	Y *(188)*
P450 11β-hydroxylase			
Rainbow trout	*Oncorhynchus mykiss*	AAD52686	LC *(72)*
Medaka	*Orizyas latipes*	AB105880	Y *(189)*
Eel	*Anguilla japonica*		Y *(190)*
3β-HSD			
Rainbow trout	*Oncorhynchus mykiss*	S72665	–
Channel catfish	*Ictalurus punctatus*	AF063835	Y *(184)*
Spiny dogfish	*Squalus acanthias*	AF005723	–
Blacktip shark	*Carcharhinus limbatus*	AF005722	–
Southern stingray	*Dasyatis americana*	AF005721	–
11β-HSD			
Nile tilapia	*Oreochromis niloticus*	AY190043	Y *(191)*
Japanese eel	*Anguilla japonica*	AB061225	Y *(191)*
17β-HSD			
Nile tilapia	*Oreochromis niloticus*		Y *(192)*
Japanese eel	*Anguilla japonica*		–
Rainbow trout	*Oncorhynchus mykiss*	AB104415	LC *(193)*
Sea squirt	*Ciona intestinalis*	AB161366	LC *(50)*
20β-HSD			
Rainbow trout	*Oncorhynchus mykiss*	AF100931	–
		AF100930	–
Zebrafish	*Danio rerio*	AF298898	Y *(185)*

Aromatase A (A) and B (B) forms are specified immediately before GenBank Accession Numbers. Studies in which expression was detectable in whole testis are indicated Y (yes), those in which testicular expression was not detectable are indicated ND (nondetectable), and those in which testicular expression has not yet been examined are indicated with "–". Within the testis, expression which has been localized to Leydig cells is indicated (LC).

Although, many of the steroid products of the testis are the same from fish to mammals (testosterone, 5α-dihydrotestosterone, estradiol, progesterone), there is considerable species diversity in the relative importance of preferred biosynthetic pathways, secreted end-products, metabolites, and conjugates (for review *see* ref. *1*).

In the primitive jawless fishes, the lampreys *Petromyzon marinus* and *Lampetra fluviati*, testosterone production is low or undetectable, but 15α- and 15β-hydroxylated C19

and C21 steroids are major products *(51,52)*. Recent studies extend this work by reporting that 15α-hydroxylated androgens, estrogens, and progestins are present in lamprey plasma and increase dramatically in response to GnRH *(53,54)*.

A prominent feature of the testes of cartilaginous fish such as sharks and skates is the high yield of several C-20β- and C-21-hydroxylated derivatives, including 17α, 20β-dihydroxypregn-4-ene-3-one, 11-deoxycortisol (S), 20β-S, 11-deoxycorticosterone, and 20β-hydroxy-4-pregnen-3-one *(55–59)*. The semen of spiny dogfish sharks is exceptionally rich in these steroids, which might be derived from steroidogenically active Sertoli cells or synthesized *in situ (60,61)* by pinched-off Sertoli cell remnants (cytoplasts) *(62–65)*. As byproducts of spermiation, Sertoli cytoplasts are abundant in shark semen, and are rich in steroidogenic organelles. The cDNA of cytochrome P450c21 has been characterized from the blacktip shark *(Carcharhinus limbatus)* adrenal *(66)*, but testicular expression was not reported.

Because most investigators begin with a bias toward the "classical" steroids, it is tempting to conclude that unconventional steroid products are merely metabolites. On the contrary, the functional roles of C-20 and C-21 hydroxylated steroids have been clearly established in teleosts (*see* "Membrane Steroid Receptors"). Gonadal 21-, 20α-, and 20β-hydroxylase activities, and plasma 11-deoxycorticosterone and other hydroxylated progestins (17α, 20β-P, 17α, 20α-P, 20β-S), are quantitatively important in males and females of several teleost fish species *(67)*. In males, 20α- and 20β-hydroxylase activities have been ascribed to spermatids and spermatozoa *(68)*, but the possibility has not been ruled out that samples are contaminated by remnants of Sertoli cells, which are sloughed into the semen in fish *(9,64)*, or that Leydig cells or Sertoli cells make a significant contribution to the C-21 steroid pool in testis, semen, and plasma *(68,69)*.

In addition to an abundance of hydroxylated progestins, teleosts are characterized by high 11β-hydroxylase and 11-oxidoreductase activities, enyzmes that are usually associated with corticosteroid biosynthesis *(70)*. 11β-Hydroxylase was cloned from the testis of rainbow trout, where strong expression was localized to Leydig cells *(71)*. Based on levels in the general circulation, natural cycles of secretion related to reproductive parameters, and in vivo or in vitro treatment experiments, it is generally believed that 11-ketotestosterone (11-KT) and 11β-hydroxytestosterone are the principal androgens in teleosts *(70,72–74)*. However, this conclusion does not account for several species in which C-11 oxygenated androgens are quantitatively unimportant: (the eel *Anguilla anguilla [75],* tilapia *Oreochromis mossambicus [76],* molly *Poecilia latipinna [77],* and catfish *Clarias gariepinus* not does it *[78]*), explain the relatively low-binding affinity of 11-KT compared with testosterone or DHT for the nuclear *(79,80)* or the membrane androgen receptor (AR) *(81)* in fish. In this context, it is important to note that synthesis and secretion *per se* is not evidence of bioactivity, even when a particular steroid is found in quantitatively significant amount and seems to correlate with biological events (*see* "Nuclear Steroid Receptors" and "Membrane Steroid Receptors"). A recent study in the rat provides direct evidence for 11β-hydroxylase mRNA and protein in rat Leydig cells, indicating it is an evolutionarily conserved characteristic *(82)*. The authors of this study suggest its role is not in regulating synthesis of bioactive steroid but, rather, in regulating glucocorticoid metabolism within the testis through local biosynthesis of endogenous inhibitors of 11β-HSD.

Although estrogen biosynthesis in testes of conventional laboratory rodents is low, and was a matter of debate for many years, comparative studies show that aromatization is a quantitatively prominent feature of the testis of certain vertebrate groups. Aromatase activity in testis-containing body segments of the protochordate amphioxous exceeds levels in ovary-containing segments by 28-fold *(83)*. Likewise, testicular aromatase activity far exceeds ovarian activity in jawless fish, for instance, the sea lamprey (*Petromyzon;* 26 vs 1 fmol/mg tissue) *(25)*. An unusual feature of the salamander *Necturus* testis is its exceptionally high estrogen biosynthetic capacity. Indeed, the specific activity of *Necturus* testicular microsomal aromatase is similar to that found in human placental microsomes *(84)* and far exceeds that measured in *Necturus* ovaries, both preovulatory and postovulatory (900 vs 50 pmol/g tissue in testis and ovary, respectively) *(85)*. Although, enzyme activity in shark testicular microsomes (0.77 fmol/min·mg protein *[122]*) is below levels reported for whole testicular microsomes of the salamander *Necturus* (120 fmol/min·mg protein) *(86)* or the mature stallion, or boar (respectively, 7 and 2.7 pmol/min·mg protein) (Canick and Callard, unpublished data), it is in the same range as that obtained from the testis of immature rats (0.30 fmol/min·mg protein) *(87)*.

Despite intense research interest in the role of estrogen biosynthesis in gonadal sex determination in bony fish *(88)*, estrogen biosynthesis is low or undetectable in the teleost testis, depending on species (*see* Table 1).

An unusual feature of teleost fish, however, is that they have extraordinarily high levels of aromatase in brain and pituitary, as compared with ovary and testis of the same species or with neuroendocrine tissues of other vertebrates *(89,90)*. A contribution of brain-formed (neuro-)estrogen to the circulating estrogen pool would explain why male teleosts have significant amounts of plasma estrogen, and why cyclic changes correlate with gonadal status *(91)*. The two *CYP19* genes (A and B) in teleosts are differentially expressed in brain (B >> A) and ovary (A >> B), and have different constitutive levels of expression (B >> A) *(44)*. Interestingly, both A- and B-subtypes are expressed in fish testis but at very low levels which do not vary with reproductive status or estrogen treatment *(92,93)*.

REGULATION OF LEYDIG CELLS

Brain–Pituitary–Gonadal Axis

In most but not all vertebrates living in the natural environment, zeitgebers like photoperiod, temperature, rainfall, and the presence or absence of conspecifics control reproduction indirectly through the brain–pituitary–gonadal (BPG) axis *(94)*. In seasonally breeding animals, it is generally accepted that the waxing and waning of Leydig cell secretions play a key role in the seasonal development and regression of male sex accessories, secondary sexual characteristics and sex behavior, and in most species peripheral effects are synchronized with germ cell production. With the possible exception of primitive jawless fish and elasmobranchs (*see* following list), reproduction in all vertebrates is regulated by the BPG axis through neural feedforward and hormonal feedback pathways *(95–97)*. This conclusion derives from work from many different laboratories, involving a wide variety of vertebrate species, and is based on the following evidence *(95–97)*.

1. Descriptions of the anatomy of the brain–pituitary complex.
2. Isolation and molecular characterization of GnRHs and gonadotropins (GTHs).
3. Experiments manipulating the BPG axis by extirpation (hypothalamic lesions, hypophysectomy, castration) and replacement (mammalian or species-specific forms of GnRH, GTH, and steroids).
4. Documentation of changes in the BPG axis during seasonal reproductive cycles and in response to environmental and social cues.

In nonmammals, as in mammals, the pituitary GTHs, luteinizing hormone (LH, LH-like, or GTHII) and follicle-stimulating hormone (FSH, FSH-like, or GTHI), regulate steroidogenesis and spermatogenesis by activating receptors expressed by Leydig cells (LH-, GTHII-Receptor) and Sertoli cells (FSH-, GTHI-Receptor), respectively. A significant feature of the GTHs and their respective receptors in lower vertebrates is that they display considerable functional overlap *(98)*. To illustrate, in salmon testis culture, GTHI and GTHII are equipotent in stimulating steroidogenesis *(99)*. If we take into account the relatively late development of Leydig cells in vertebrate evolution, and their increasing importance in steroidogenesis, it is not surprising to observe corresponding evolutionary changes in the specificity of GTHs, their receptors, and subfunctions.

In contrast to the HPG axis in bony fishes and higher vertebrates, the existence of a functioning HPG axis in cyclostomes and elasmobranchs is unclear. It is relevant here that Leydig cells are undifferentiated in these two groups (*see* "Primitive Fish" and "Cartilaginous Fish"). Both cyclostomes (lampreys and hagfish) and elasmobranchs (sharks, skates, and rays) have several forms of GnRH, but cloning of gonadotropin subunits (α- and FSH-like and LH-like β-subunits) has been successful only in the dogfish shark *Scyliorhinus (100)*. Although the cellular targets and biological functions of these GTHs have not yet been reported, the early studies of Jenkins and Dodd in the same species indicate that spermatogenesis, but not steroidogenesis, is pituitary-dependent *(101)*. This study showed that plasma testosterone levels do not change seasonally, nor is there an effect of hypophysectomy *(102–104)*. By contrast, spermatogenesis had a clear seasonal component, and hypophysectomy induces spermatogenic arrest. Nonetheless, effects are more subtle than in mammals, because hypophysectomy does not entirely deplete the germ cell population. Instead, hypophysectomy mimics seasonal arrest by interrupting the transition of spermatogonial clones into meiosis *(101)*. Affected clones then undergo apoptosis and are removed from the progression *(105–107)*. These data suggests that the FSH-like functions of the HPG axis on spermatogenesis might have evolved in advance of the LH-like effects that control peripheral steroid secretion, which, in turn, coevolved with Leydig cells.

Relation to Spermatogenesis

Available evidence indicates that high intratesticular steroid levels are required for production and development of mature male gametes throughout the vertebrata. In addition to their role in secreting steroids into the peripheral circulation, it is generally accepted that Leydig cells provide a steroid-rich germ

cell environment, with relatively minor qualitative contributions from Sertoli cells and germ cells themselves. However, there are exceptions, even in mammals. In animals, in which spermatogenesis is temporally dissociated from development of the peripheral reproductive system (e.g., certain teleost fish *[108]*, reptiles *[38,57,109]*, and bats *[110]*), and animals in which fully differentiated Leydig cells are absent (e.g., jawless and cartilaginous fishes), Sertoli cells have the primary responsibility for maintaining steroid levels in close proximity to germ cells. In all vertebrates, structural and functional changes in Leydig cells are coordinated with germ cell development and, in turn, with Sertoli cell status, implying multidirectional cell–cell interactions. Compared with conventional laboratory mammals, coordination among the different cell types is much more readily documented in animals with "zonal testes," in which there is a clear spatial separation of different germ cell stages and associated Leydig and/or Sertoli cells, for example, cartilaginous fish; teleost fish (restricted form); and urodele amphibians. The salamander *Necturus* and the dogfish shark *Squalus* have been studied in some detail *(111,112)*.

In *Necturus*, Leydig cells are undifferentiated in regions adjacent to tubules with germinal clones in spermatogonial, spermatocyte, and mature spermatid stages of development. Tissues dissected from these regions have correspondingly low levels of steroidogenic enzymes. However, in regions where spermiation has already occurred, Leydig cells hypertrophy, develop a massive amount of smooth reticulum and other steroidogenic organelles, and display a dramatic increase in cytochrome P450, hydroxylase/lyase and aromatase activities *(26,28,30,87)*. Because spermiation is limited to tubules at the distal (mature) pole of the testis, highly differentiated Leydig cells are restricted to a subcapsular "glandular" zone. Moreover, the spermatogenic wave passes from posterior to anterior; thus, there is a distinct correlation between the state of differentiation of Leydig cells and enzyme activity, even within the glandular region *(28,30,113)*. Exactly what regulatory factors and effector pathways are involved in local control of Leydig cells have not been studied further, but the situation in *Necturus* is reminiscent of events occurring in rat Leydig cells after damage to a localized region of the seminiferous epithelium *(114–116)*, and are consistent with data in rats showing that tubular concentrations of immunoreactive androgen *(117)* and the size of adjacent Leydig cells *(118)* are greatest just before sperm release (Stages VII and VIII of the seminiferous epithelial cycle).

The situation differs somewhat in the dogfish shark *Squalus*, in which differentiated Leydig cells are absent and Sertoli cells are the primary steroidogenic element *(15,119–121)*. Consistent with maturation-related changes in Leydig cells in rats and *Necturus*, dogfish shark Sertoli cells undergo dramatic stage-related increases in size, cytological features, and in steroidogenic enzyme activities *(15,119,120)*. To illustrate, Sertoli cells associated with stem cells and spermatogonia are small, with few steroid secretory organelles, but at the onset of spermiogenesis they develop increasing amount of smooth reticulum *(15)*. Key enzyme activities leading to androgen biosynthesis (3β-HSD, 17α-hydroxylase/lyase) increase progressively from less mature to more mature stages of spermatogenesis *(122)*, and similar increases in 21-hydroxylase activity are observed *(123)*. By contrast, aromatase activity is highest in regions with spermatocytes and round spermatids (meiotic stages), whereas 5α-reductase activity has a different pattern *(124)*. Biochemical analyses have now been confirmed by Northern analysis and reverse transcriptase-polymerase chain reaction analysis of staged tissues using species-specific primers or probes for aromatase and 17α-hydroxylase, indicating that regulation is at the pretranslational level (Engel et.al., unpublished data).

NUCLEAR STEROID RECEPTORS

Nuclear receptors for all major steroid hormone classes (androgen, estrogen, progestin, corticosteroid) have been identified in the testes of representatives of all major vertebrate groups (for review *see* refs. *125* and *126*) indicating that steroids synthesized *in situ* act through genomic mechanisms to regulate testicular functions, and that this mechanism of control is ancient and highly conserved. Initially, identification of steroid receptor was based on their binding activities and other physicochemical characteristics, but in the last decade cDNAs of nuclear steroid receptors have been isolated by homology cloning approaches in many different nonmammalian groups (Fig. 1; for review *see* refs. *125–127*). Based on multispecies sequence comparisons and phylogenetic analyses, it has been postulated that the estrogen receptor (ER) is the ancestral steroid receptor *(125,126)*. Teleost fish often have multiple gene loci of a given steroid receptor subtype where most other vertebrate groups have a single locus. Thus, co-orthologs of mammalian ERβ in teleosts are ERβa and -βb *(128)*. Similarly, two AR genes and two progestin receptor (PR) genes have been identified in at least one teleostean species, the

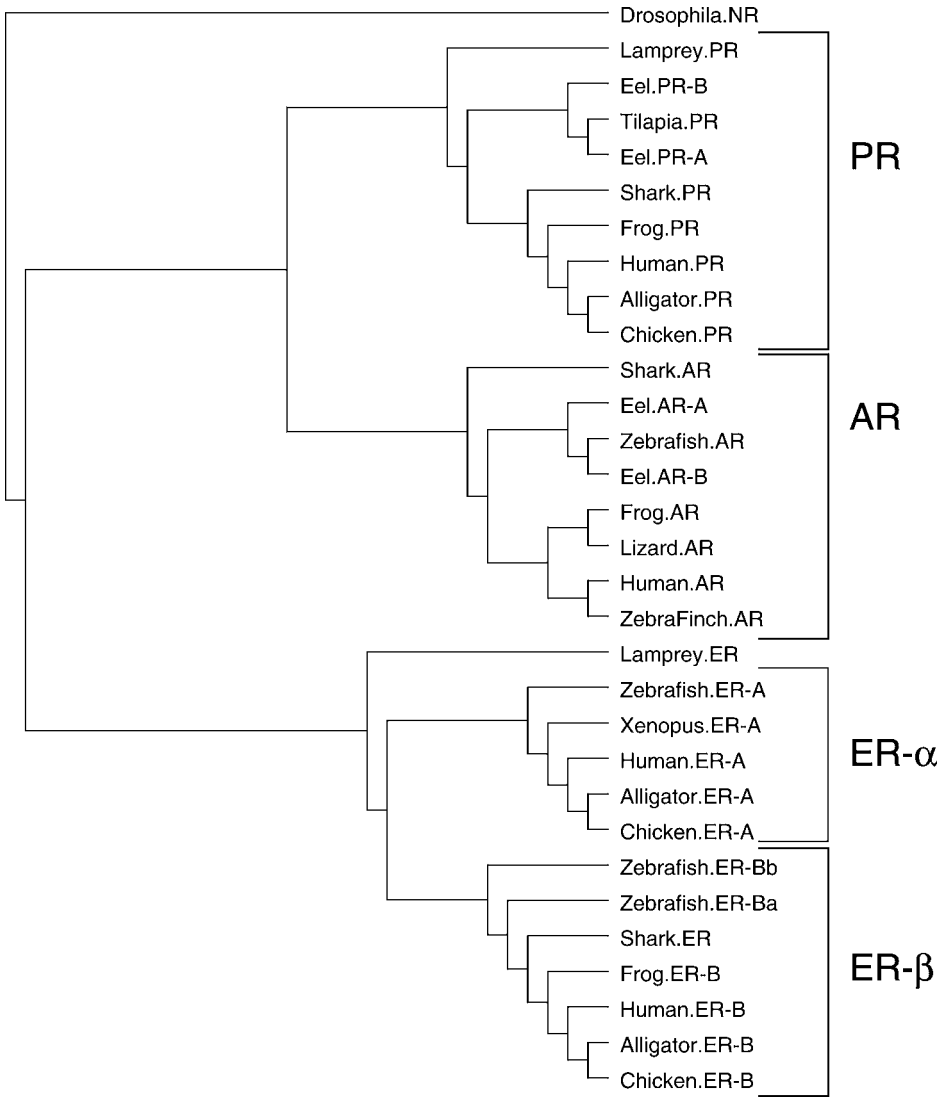


Fig. 1. Phylogenetic tree of nuclear steroid receptors using deduced amino acid sequences of representative vertebrates and CLUSTAL X 1.81. GenBank Accession numbers: human (PR, NP_000917; AR, 105325; ERα, AAA52399; ERβ, AAC05985); chicken (PR, AAA49013; ERα, P06212; ERβ, NP_990126); zebra finch (AR, AAM96699); alligator (PR, BAD08350; ERα, BAD08348; ERβ, BAD08349); anole lizard (AR, AAF28356); African clawed frog (ERα, NP_988866; ERβ, AAQ84781); bullfrog (PR, AAN63590; AR, AAP85538); zebrafish (AR, AAS80170; ERα, AAK20929; ERβa, AAK16740; ERβb, AAK16741); eel (PRα, BAA89539; PRβ, BAB85993; ARα, BAA75464; ARβ, BAA83805), tilapia (PR, BAC77019); dogfish shark (PR, DQ223103; AR, AAP55843; ER, AF147746); lamprey (PR, AAK20931; ER, AAK20929), drosophila pOT2 nuclear receptor (AA821160).

eel *Anguilla (129)*. Duplicates often have separate expression domains and subfunctions, which facilitates regulatory and functional analyses. Not all receptors are duplicated in teleosts (e.g., ERα), which raises interesting questions about selection pressures that affect specific loci. Alternative promoters and splicing mechanisms further increase the diversity of nuclear receptor isoforms, but these variants have been only superficially characterized in nonmammalian vertebrates. Moreover, the exact cellular location and stage-related changes in receptor expression have rarely been studied.

Androgen Receptors

In the zonal testis of the dogfish shark, AR binding activity is concentrated in regions with spermatocysts in premeiotic stages *(130)*, implying a significant role in processes specific to early spermatogenesis. The AR cDNA has been cloned from the testis of the shark *Squalus*, and real time quantitative polymerase chain

reaction analysis (Engel and Callard, unpublished data) confirms the stage-related pattern previously found by binding analysis. Immunolocalization using a human AR antipeptide antibody reveals localization in Sertoli cell nuclei of premeiotic cysts, and in cells of the collecting ducts interspersed among spermatocysts in this region *(6)*.

Paradoxically, teleostean AR have relatively low affinities for 11-KT, even in fish with high levels of plasma 11-KT, the presumptive teleost androgen; by contrast, T and DHT are good ligands *(80,81,131,132)*. Moreover, T and 11-KT have similar activational activity on AR reporters *(133,134)*. Alternative hypotheses for 11-KT's effectiveness when administered in vivo or in vitro is that 11-KT, as compared with T or DHT, is less readily converted to inactive metabolites. Alternatively, unchanged 11-KT could have more availability for AR-binding than T because (a) it is not a substrate for aromatization, an exceptionally active pathway in neuroendocrine tissues of teleosts and (b) it has very low affinity for plasma sex-steroid-binding protein *(80)*. There is no evidence to date of an 11-KT specific receptor. To our knowledge, the androgen actually associated with target cell nuclei in teleosts has not been identified.

A single AR gene has been cloned from the bullfrog *(135)* and the whiptail lizard *(136)*, but tentative identification of a second AR gene has been reported in *Xenopus (137)*, which might reflect polyploidy in this species *(138)*. A T-binding macromolecule having the basic features of a classical nuclear AR has been characterized in *Necturus* testis *(139)* and is present both in immature germinal regions, where Leydig cells are sparse and undifferentiated, and in subcapsular glandular regions, where Leydig cells are highly differentiated but seminferous lobules are regressed. The inference is that AR have multiple locations in *Necturus*, in Sertoli cells, Leydig cells, and germ cells, depending on spermatogenic stage. Testicular AR-binding activity has also been characterized in the testis of the frog *Rana (140)* and the lizard *Podarcis (141)*, where levels are highest during periods of active spermatogenesis and higher in interstitium than in tubules.

Estrogen Receptors

Despite a continuing debate over the physiological role of estrogens in male reproduction, conservation of ER binding activity and mRNA in the testis of a wide range of vertebrate species indicates some adaptive significance *(113,141–145)*. The dogfish shark *Squalus* has been useful in providing some clues to the estrogen-sensitive stage of spermatogenesis. In this species, ER binding is highest in regions with stem cells and spermatogonia and virtually nondetectable in regions with mature germ cells *(122,142)*. Stage-related ER distribution has been confirmed by cloning and analysis of shark specific ER mRNA (Engel et.al., unpublished data). The topographic relationship of ER in premeotic regions and aromatase meiotic regions, (which is upstire in the vascular pathway), reinforces the view that estrogen has an autocrine role in signaling the advance of spermatogenic development.

All three teleostean ER genes are expressed in the testis of goldfish *(146)* and killifish *(146a)*. In killifish testis, expression levels are $\beta a > \beta b > \alpha$. In the channel catfish, ERα and ERβ are localized to secondary spermatocytes, spermatids, and mature sperm *(147)*, but in rainbow trout a homologous antibody immunolocalized ERα to interstitial fibroblasts, presumptive Leydig cell precursors *(148)*. In *Necturus*, cytosolic ER (unoccupied, unactivated) are highest in glandular tissue comprising Leydig cells only, but when nuclear ER (occupied, activated) are measured, levels are two- and threefold higher in regions where Leydig cells are undifferentiated and tubules are in the spermatogonial/spermatocyte stages of development *(30,113)*.

Progestin Receptors

Binding activity consistent with the presence of nuclear PR has been described in testicular extracts of the dogfish shark *(130)*. In this species, PR have a distinctly different stage-related distribution (mature >> immature, two- to sevenfold differential) than AR (immature >> mature, two- to fourfold differential) *(130)*, indicating distinct receptor moieties, and association of PR with processes such as spermiogenesis and sperm release. Cloning and mRNA analysis of shark PR generally, confirms distribution based on binding activity, but reveals significant expression in premeiotic stages as well (Engel and Callard, unpublished data). Of the two PR cloned in eels, both are expressed in the testis (PR2 > PR1) *(149)*, but PR1 has higher affinity for 17α, 20β-dihyroxy-4-pregnen-3-one than for progesterone *(150)*.

MEMBRANE STEROID RECEPTORS

In the female Atlantic croaker, among other teleosts, it has been established that 20β-S binding to a novel seven transmembrane domain, G protein-coupled receptor (mPR) mediates meiotic maturation of oocytes *(151)* (*see* below). In the male of the species, 20β-S upregulates sperm motility by a short latency mechanism *(152)*, presumably through the mPR protein localized on the sperm membrane *(153)*. This progestin upregulates croaker sperm motility by inducing rapid increases

in intrasperm cAMP concentrations and influx of calcium through L-type voltage-sensitive calcium channels *(152,153)*. Significant transcript levels of an ortholog of mPR is expressed in human testis as well *(154)*, suggesting that it could mediate rapid progesterone induction of processes such as capacitation, hypermotility, and the acrosome reaction in mammalian sperm, all of which involve rapid calcium influx and increases in cAMP levels *(155)*.

TOXICOLOGY AND ENDOCRINE DISRUPTION

Epidemiological research indicates that male-mediated infertility and negative effects of paternal toxicant exposures on children's health could be increasing *(156)*. The list of chemicals known or suspected to be spermatotoxicants continues to expand and includes endocrine disrupting chemicals (EDCs). By their interaction with hormone receptors, EDCs have the potential to disrupt critical hormone-regulated processes of reproduction and development. Because of effluent discharges and runoff, bodies of water are particularly susceptible to contamination. Although, terrestrial organisms (including man) are vulnerable through drinking water and food web bioaccumulation, aquatic and marine organisms have the highest exposure risk, and present a unique opportunity to serve as sentinels. To illustrate, male fish located downstream of estrogenic effluent discharges have detectable plasma levels of vitellogenin, a yolk protein precursor specific to oviparous vertebrate females, reduced gonadosomatic index (GSI), and reduced plasma 11-KT levels *(157)*. Laboratory administration of 17α-ethinylestradiol similarly affects vitellogenin and GSI, but also results in elevated plasma estrogen levels, and increased testicular apoptosis, which includes Leydig cells, Sertoli cells, spermatocytes, and spermatids *(158,159)*. In another example, a killifish population exposed over multiple generations to high levels of PCBs at a Superfund site (New Bedford Harbor MA, NBH) has continued to reproduce despite evidence of estrogenic and dioxin-like effects, deficits in the HPG axis, and reduced GSI *(160–162)*. This population, and fish at other polluted sites, are a valuable resource for understanding genetic and physiological adaptations to long term pollutant exposure effects on reproduction.

In addition to their presence in the aquatic environment, elasmobranchs have an additional advantage as a sentinel species, namely, the orderly arrangement of successive stages in the spermatogenic progression. The advantageous testicular organization in sharks, skates, and rays increases the likelihood of recognizing even subtle spermatotoxicant effects, and at the same time facilitates identification of affected stages, cell types, and processes. Research in this laboratory has used the dogfish shark to show that exposure to cadmium in the environment or in the laboratory targets spermatogonial stages preferentially, where it increases the percentage of spermatogonial clones that become apoptotic *(163)*. A current goal of research in this laboratory is to develop the little skate *(Leucoraja erinacea),* a nonmigratory species at the NBH Superfund site, as an *in situ* indicator of spermatotoxicity *(164)*. Additionally, the recent breakthrough in identifying steroid membrane receptors, first in fish and then in other vertebrates, promises to open a new focus of endocrine disruptor research *(152)*.

ADAPTIVE SIGNIFICANCE OF LEYDIG CELLS IN EVOLUTION

The evolution of testes with higher organizational complexity and more diverse cellular repertory, including Leydig cells, is just one aspect of a multitude of reproductive adaptations which accompany animal evolution *per se*. These adaptations are a consequence of selection pressures operating on reproductive success and include:

1. Migration of species into a wider range of habitats (e.g., terrestrial, freshwater, temperate).
2. Prediction and exploitation of optimal conditions in a single environment (e.g., cycles of temperature, photoperiod, or resources).
3. Improved efficiency of mate identification, mating strategies, and offspring survival (e.g., internal fertilization).
4. Responses to increased complexity of female reproductive processes (e.g., viviparity, delayed implantation, parental care).

All these challenges involve mediation of environmental change by the male BPG axis. It is proposed here that Leydig cells evolved primarily for the purpose of secreting steroids into the general circulation as a means of controlling and integrating multiple responses at extratesticular sites and secondarily usurped the role of Sertoli cells in maintaining the steroid *milieu* of the germinal compartment.

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IV Regulation

16 The Luteinizing Hormone Receptor

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SUMMARY

Both major and incremental advances in the past decade have provided important insights into the structure, function, and regulation of the luteinizing hormone receptor (LHR) and its gene. The availability of the crystal structure of the extracellular domain of the follicular stimulating hormone receptor–hormone complex, and its applicability to other members of the gonadotropin receptor family, has facilitated prediction of the localized areas of specific interaction between the LHR and its cognate hormone. This has been supported by the incorporation of information derived from mutational and chimeric studies. The nature of the structural and molecular changes involved in the initiation and propagation of signaling is beginning to be revealed by the various models. It is likely that components of each domain of the LHR, the hinge region of the extracellular domain, and the LH/human chorionic gonadotrophin α-1,3 loops participate in the receptor activation. Naturally occurring and created mutations have provided fertile ground for modeling, leading to plausible proposals for the regions of the receptor that participate in receptor coupling functions. The knockout mouse model has demonstrated the lack of participation of the hormone/receptor in fetal gonadal development, in contrast with the situation in the human, where the essentiality of the LHR is exemplified by Leydig cell hypoplasia. The association of an LHR polymorphism with breast cancer age of onset and severity has been documented. Significant advances have been achieved in studies on *LHR* gene structure and regulation of transcription. Two independent mechanism of repression/derepression have been identified. Studies on the epigenetic control of LHR transcription have revealed the combined importance of promoter methylation status; and changes in histone acetylation/methylation in the association/release of inhibitory complexes from the promoter, and recruitment of the preinitiation complex and polymerase II. Furthermore, major advances await elucidation of the participation and molecular mechanisms of signaling pathways involved in the transcription of the LHR.

Key Words: Breast cancer; degradation; testotoxicosis; function; Leydig cell hypoplasia; LHR; polymorphism; structure; trafficking; translation; transcriptional regulation.

INTRODUCTION

The leuteinizing hormone receptor (LHR), a member of the G protein-coupled receptor (GPCR) family is essential for normal sexual development and reproductive function. The LHR is expressed primarily in the gonads, but is also found in nongonadal and cancer tissues. LH acts through membrane LH/human chorionic gonadotrophin (hCG) receptors in Leydig cells to maintain general metabolic processes and steroidogenic enzymes to regulate the production of androgens *(1)*. In the ovary, LH promotes follicular development at stages beyond early antral follicles, including the formation of preovulatory follicles and corpora lutea, and enhances steroidogenesis in granulosa and luteal cells *(2)*. Testicular LHRs are expressed during fetal life, postnatally, at puberty, and throughout adult life. LHRs are not detectable in the fetal ovary but are expressed in early neonatal life. hCG which is secreted from the placenta from the time of implantation and is structurally similar to LH, has a longer half-life and binds to the LHR with higher affinity than LH *(1)*. Both hormones, together with follicular stimulating hormone (FSH) and TSH, belong to the GPCR subfamily of glycoprotein hormone receptors. These exist in their active conformation as heterodimers with a common α-subunit and a dissimilar β-subunits that confers biological specificity on the individual hormones. Both subunits contain a cystine knot motif core and extended hairpin loops. The two subunits are linked by noncovalent interactions stabilized by a β-cysteine loop that forms a seatbelt encircling loop 2 of the α-subunit

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(3,4). Expression of LHR is induced by hCG during fetal life and by FSH and LH postnatally.

The steroidogenic actions of LH are exerted primarily through cyclic adenosine monophosphate (cAMP)-mediated events in the gonads *(5,6)*. In the testis and ovary, agonist-activated LHR are coupled primarily to guanylnucleotide stimulatory protein (Gs), leading to stimulation of adenylate cyclase and phosphorylation of intracellular proteins through activation of protein kinase A *(7)*. However, the phosphoinositide (PI) signaling pathway is also operative, and agonist activation of LH/hCG receptors promotes PI hydrolysis and calcium signaling *(8,9)*. Pulsatile endogenous or exogenous LH secretion or single low-dose treatment with LH or hCG maintains LHR levels and steroidogenic enzymes in adult Leydig cells. However, major sustained increases of LH levels, or endogenous elevations of hCG (e.g., choriocarcinoma), or a single pharmacological dose of LH or hCG, can cause down-regulation of the LHR and desensitization to the hormonal signal *(1,5)*. Unlike the adult rat testis, the fetal and immature rat testes are refractory to desensitization. However, this can be induced by exposure of immature cell cultures to gonadotropin. This might result in part from the lack of direct impact of the hormone in the fetal testis, as has been recently revealed by studies in LHR null mice, which indicated that sex differentiation in the rodent is not dependent on LH action *(10,11)*.

At birth, the LHR null mice displayed testes, ovaries, and accessory organs that were not different from the wild-type. In the absence of the LHR, the fetal testis can produce testosterone and anti-Mullerian hormones that are essential for intrauterine masculinization *(10,11)*. It seems that neither LH/LHR action is required, or can be adequately compensated by other hormones or factors during fetal life. However, major changes were found after birth, when testis growth and descent, and the growth of accessory organs, are significantly inhibited. Although, gametogenesis completed meiosis, it did not proceed beyond the round spermatid stage. It is conceivable that this is related in part to the reduction or absence of expression of the gonadotropin-regulated testicular helicase *(12)*. This enzyme, which is upregulated by gonadotropin in vivo and in vitro through the action of androgen, is essential for elongation of spermatids and progression of spermatogenesis. Mice null for gonadotropin-regulated testicular helicase are sterile with complete spermatogenic arrest at steps 7/8 of round spermatids *(12)*. Androgen administration restores the testicular function and fertility in 15% of the LHR null mice. However, 46% of the testosterone-treated animals displayed normal sexual behaviour *(13,14)*. The pan-LHR null mice model has not provided the expected insights into the role of LHR function in nongonadal tissues. In contrast to the murine, in humans the activation of fetal LHR by hCG *in utero* is essential for expression of the male phenotype at birth, as revealed in patients with inactivating mutations of the LHR. Moreover, there is a correlation with receptor activity resulting from the nature of the mutation and the phenotype *(7,15,16)*.

It has been suggested that FSH can influence Leydig cell function indirectly through Sertoli cell-derived factors, including cytokines and growth factors. Treatment of hypophysectomized immature rats with pituitary FSH, caused hyperplasia and hypertrophy in Leydig cell, as well as enhanced steroidogenic responses *(17)*. Moreover, studies using neutralizing antibodies have suggested a stage-specific function for FSH in the regulation of Leydig cell development *(18)*.

The trophic actions of growth hormone, given alone or with prolactin, promote receptor expression and prevent receptor loss following hypophysectomy. In growth hormone-receptor-null mice, LH-induced testosterone secretion and Leydig cell volume were significantly reduced because of the decreased number of testicular LH and prolactin receptors *(19)*. Insulin like growth factor (IGF)-I, which is undetectable in growth hormone-receptor-null mice, is a major modulatory factor in Leydig cell function and increases Leydig cell steroidogenic responsiveness to hCG by increasing LHR expression *(20)*. IGF-I-null mice are infertile dwarfs-with delayed Leydig cell development and reduced serum testosterone levels *(21)*. IGF-I also increases LH-binding and LHR messenger RNA (mRNA) in an immortalized murine tumor cell line by increasing the stability of LHR transcripts *(22)*.

Prolactin treatment has been shown to increase the number of Leydig cells and LHR in hypophysectomized immature rats *(23–25)*, and induction of hypoprolactenemia suppressed testicular LHR levels *(26,27)*. In men, hyperprolactenemia is associated with azoospermia and hypogonadism, and has direct inhibitory effects on Leydig cell steroidogenesis *(28,29)*. The nature of such discrepant findings might relate to the functional state of the Leydig cells and concentration of the hormone, because studies on MLTC1 cells (mouse tumor Leydig cells) demonstrated biphasic regulatory actions of PRL with low doses stimulating and high doses inhibiting LHR expression *(30)*.

The cDNA of the LHR was cloned from pig and rat testes, as well as mouse and human libraries *(31–34)*, and its gene structure has been defined in rat, human, and mouse *(35–38)*. Considerable information about the

structure–function properties of the receptor has been derived from endogenous and engineered mutations and modeling studies. The recent elucidation of the crystal structure of the extracellular domain (EC) of the FSH receptor in its liganded form has provided valuable insights that are also applicable to LHR structure. Also, recent studies have begun to elucidate the mechanisms of repression and derepression that govern the *LHR* gene expression.

STRUCTURE OF THE RECEPTOR

The cDNA for the LHR encodes a 75 kDa protein that contains 674 amino acids *(31–34)*. The receptor is a sialoglycoprotein made up of two functional units—the extracellular hormone-binding domain, which binds LH and hCG with high affinity *(35,39)* and the seven membrane transmembrane (TM) helices connected by extracellular/cytoplasmic loops (IL)/cytoplasmic module (Fig. 1). This membrane-associated structure transduces the signal initiated in the EC by coupling to G proteins, and is also involved in receptor recycling. The LHR contains a cleavable N-terminal signal sequence that directs its insertion in the endoplasmic reticulum. The size of the mature receptor is 80–90 kDa, of which about 15 kDa are derived from carbohydrate chains located in the EC at six potential glycosylation sites (AsnXxxSer/Thr). Five of these have carbohydrate chains at N152, 173, 269, 277, and 291, whereas the N77 (rat) site lacks the glycosyl residue *(40,41)*. However, other studies have indicated that all sites are glycosylated *(42)*.

The LHR contains *N*-linked glycosyl residues of the complex type, with terminal sialic acid on the EC. Based on the alignment of the ribonuclease inhibitor-A leucine-rich repeat, the carbohydrate chains at N^{173} and N^{152} are located on the outer surface of the receptor, but not within the LHR-binding domain *(7,43)*. These appear to be involved in the refolding and conformational stability of the mature receptor and in intramolecular folding of the nascent receptor, rather than in hormone-binding. Renaturation occurs as long as the receptor bears the proximal *N*-acetylglucosamine residues on Asn^{152} and Asn^{173} and the four cysteines in exon 1 *(41,44)*. The hydrophobic leucine-rich repeat region that constitutes the binding domain is intercalated between cysteine-rich domains in exons 1 and 9. This repeat, which consists of the first 250 residues of the EC, is separated by a linker sequence of variable length in the members of the family.

Individual mutations of all Cys residues in exon 1 ($Cys^{8,12,14,22}$ rat, corresponding to $Cys^{5,9,11,19}$ human) to serine revealed that they are essential for hormone-binding *(44)*. The four Cys residues in exon 1 of the FSH receptor (FSHR) are disulfide bonded *(45)*. The LHR contains three cysteine residues that are not conserved in the FSR and TSHR, two of which (Cys^{109} and Cys^{134}) are located in domains implicated in LH/hCG-binding by chimeric and mutational studies of the LHR *(41,46)*. Like other members of the GPCR superfamily, the LHR contains Cys residues in exoloop (EL)1 and EL2, which form an intramolecular disulfide bridge that stabilizes the helical TM structure. A number of amino acid residues (Cys and non-Cys residues in exons 7–11 of the LHR) are required for membrane insertion and receptor processing *(44,47–49)*.

The C-terminal intracellular sequence contains several serines and threonines that are amenable to phosphorylation by protein kinase A. Serines 635, 639, 649, and 652 are phosphorylated on hCG stimulation or phorbol ester treatment *(50)*. This domain also contains $Cys^{621, 622}$ (rat), $Cys^{618, 619}$ (human) that are palmitoylated *(51,52)*. Mutations of these Cys residues does not affect ligand-binding, cAMP production *(51,53)* or PI signaling *(54)*, but increases the rate of hCG-induced receptor internalization and decreases recycling *(51,55)*. This has been attributed to enhanced interaction of the mutant receptor with the arrestin-mediated internalization pathway *(54)*. Furthermore, palmitoylation-deficient mutant receptors are more prone to phosphorylation, which in turn prevent their efficient recycling from endosome to the cell surface and divert the LHR to the lysosomal degradative pathway *(54)*. In contrast, the reduced phosphorylated wild-type receptor (palmitoylated receptor) might be more susceptible to dephosphorylation and thus amenable to recycling to the cell surface. It is of interest that the naturally occurring $D^{578}H$ mutant, which is defective in palmitoylation, also, shows decreased recycling *(55)*. However, other studies have indicated that phosphorylation does not appear to participate in sorting, as mutation of all phosphorylation sites to alanine did not change the postendocytic fate of the human LHR (hLHR) *(56)*. Other elements that are involved in recycling include a GT motif and a Cys and Leu in the C-terminal tail *(56)*. Like most GPCRs, the hLHR is internalized and rapidly recycled back to the plasma membrane. In contrast, the rodent and porcine LHR are largely directed toward a lysosomal degradation pathway. However, transfer of the GT motif present in the C-terminal tail of the hLHR to the rLHR promotes the postendocytotic recycling of the agonist–internalized rLHR complex. Although, the GT motif is not directly involved in recycling, its presence might permit the exposure of other unidentified motifs that are more directly involved in recycling *(57)*. The Leu^{683} and

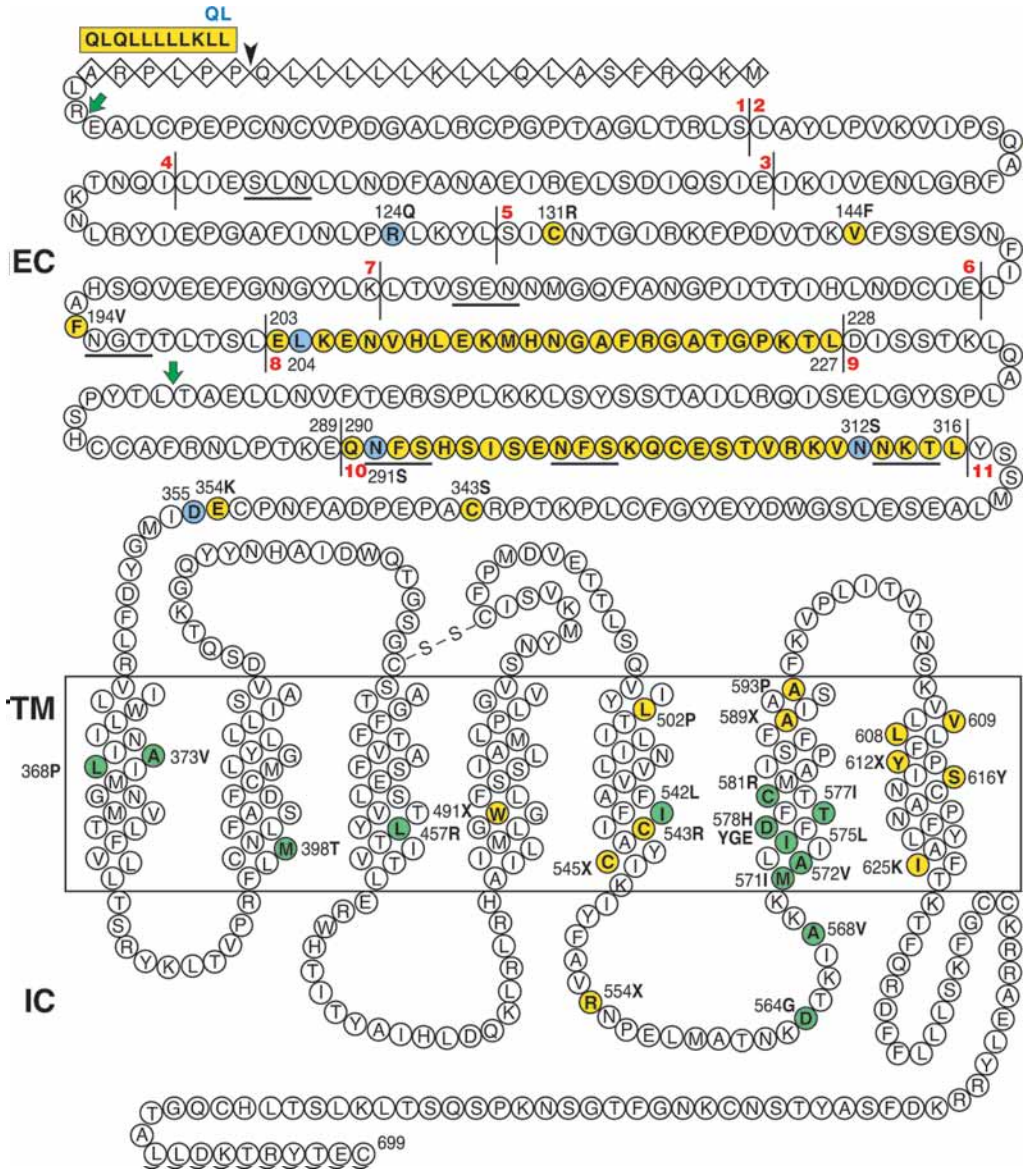


Fig. 1. The deduced amino acid sequence of the hLHR *(33)*. Exon divisions are represented by vertical lines and exons are numbered 1–11 in red. Amino acid residues corresponding to the putative cleavable signal peptide (diamonds) and those of the mature peptide (circles). Underlined: *N*-glycosylation sites. Leucine- rich motif region (region between open arrows). Hinge region aa 273–363. Activating mutations noted as black circles in FMPP-autosomal dominant and/or sporadic male-limited precocious puberty or other. Inactivating mutations or deletion of exon 8 or 10 or insertion of 33 bp at nucleotide 18, observed in LCH (gray squares). Polymorphisms noted as open triangle. LQ insertion (black arrow-head). Mutated amino acid is indicated next to the normal amino acid residue. X: stop codon. For conversion to the human sequence and rat sequence amino acid mature peptide substract 26. The region relevant in coupling functions coincides with the highest abundance of naturally occurring mutations. EC, extracellular domain; TM, transmembrane domain; IC, intracellular domain. (Please *see* color version of this figure in color insert following p. 180.)

terminal Cys^{699} residues are also necessary for recycling of the LHR *(56)*.

A high proportion of LHR molecules in the gonads and nervous system and in expression systems, exists as immature forms *(58–60)*. The immature LHR of molecular radius (Mr) 73,000 contains high mannose-type *N*-linked glycans, of which only 20% were found to mature to the Mr 90,000 sialylated *N*-linked glycan cell-surface receptors. The majority form complexes, which are retained in the ER and undergo proteosomal degradation and proteosomal blockade increases the proportion of LHR-binding hormone *(61)*. Early reports indicated that agonist-activated LHR are clustered within high-molecular weight structures in the cell membrane

(62–64). More recent studies have shown that hormone binding to the rat LHR causes its translocation from the bulk membrane into lipid raft structures. However, disruption of the rafts reduced, but did not abolish LH signaling *(65)*.

The recently determined crystal structure of the FSH molecule bound to the EC of the FSH receptor has revealed that the latter is dimeric in the hormone–receptor complex *(45)*. However, FSH does not participate in the physical formation of the complex, which is derived by the association of the two receptors and their bound FSH molecules. The availability of this structural data provides a theoretical framework for the understanding of complexes of the other members of the receptor family. Specifically, dimerization of the LHR could be predicted using the FSHR crystal structure as the template. In addition, early studies demonstrated that the native mature receptor was present as monomers/dimers and that both forms bound the hormone *(66)*. Self association of LHR on hormone-binding was revealed by fluorescence-resonant energy transfer method *(67)*, and coimmunoprecipitation of differentially tagged hLHR in living cells showed LHR dimerization that was enhanced in the presence of hormone *(68)*.

The crystal structure of the complex shows that the FSHR EC is shaped as a slightly curved tube formed by 10 leucine-rich repeats, which are irregular in length and conformation and contain an N-terminal cysteine-rich cluster that forms two disulfide bridges (Fig. 2). Leucine-rich repeats 7–10 displayed the signature horseshoe-like curvature, whereas repeats 1–7 arrange are almost flat. These features are probably conserved in the LHR and TSHR structures. The hormone binds to the inner concave surface of the curved receptor in a "hand-clasp" fashion and makes contacts with all 10 parallel β-strands and the loops C-terminal to the β-sheet. Both subunits are involved in determining the specificity of binding to the individual receptor through both charge and conformational properties, the latter being more attributable to the α-subunit. FSH binds to the receptor by fitting into the binding-site through its β-subunit second loop, leading to changes in conformation and reshaping of the α-subunit loops that maximize the protein–protein interaction. The receptor surrounds the midportion of the FSH molecule and interacts with the C-terminal segments of both subunits and the α- and β-L2 loops. Mutational studies have demonstrated that amino acids in the α-subunit carboxyl terminal tail are essential for receptor-binding *(69)*. Structural studies indicate that rotation of the α-tail caused its engagement with the receptor at the hormone–receptor interface. The β-L1,3 loops projects out from the C-terminal tip of the receptor inner sheets and the α-L1,3 loops protrude away from the base *(45)*. The large interface of the complex has a high charge density, with electrostatic potential surfaces, which are predominantly positive for the hormone and negative for the receptor. The interface is devoid of carbohydrate groups contributed by the hormone or receptor, as all of these are distant from the interface. Although, glycosylation of the FSH-α-subunit is important for optimal signaling, this has not been implicated in hormone-binding *(70)*. Sulfation of Y^{318} of the FSHR, which is close to the membrane and conserved in all members of the glycoprotein receptor hormone family, is required for high-affinity-binding *(71)*. The crystal structure of the complex was found to compare fairly closely with the proposed model of hCG-binding to ribonuclease inhibitor *(72)*.

Just as the FSH and hCG molecules are structurally similar and possess identical species specific α-subunits, the structure and conformation of their receptor-binding sites also share many common features. These properties are consistent with the concept of a general-binding mode between glycoprotein hormones and their specific G protein-coupled receptors. This is further indicated by the presence of common structural motifs within the shared binding surfaces of the gonadotropin receptor complexes. Also, by the conservation of amino acids involved in the interaction within the glycoprotein hormones and their receptors. Many of these pairings involves complementary charged groups—including salt bridges and other charge-dependent interactions—that contribute to interactions with hormonal α-chain, and also mediate contacts with conserved β-chain residues and the receptor. This latter accounts for the importance in receptor-binding of salt–bridge interaction between K^{104} of the FSHR with the common aspartate of FSH-β (D^{93}, FSH; D^{99} hCG) *(73,74)*. Furthermore, an interaction between aromatic residues in FSH-α (Y^{88}) and its receptor (Y^{124}) might also occur during hormone-binding to the LH and TSH receptors (Y^{127}, F^{130}) *(45,75)*. Salt bridges connect D150 and D^{153} of FSHR and basic residues of the α-subunit K^{91} and K^{51}. This explains the loss of function by charge reversal mutation of the corresponding residues in LHR E^{132} and D^{135} *(76)*, and alanine mutations of FSH-α K^{51} and K^{91} *(77,78)*.

Specific selective regions of receptor-binding have been localized to a unique seat belt segment (amino acids 89–105) of the β-subunit *(79–81)*. This segment is at the interface between the α-L2 loop and the C-terminus (CP) of the FSH-α. In addition, the tip of the

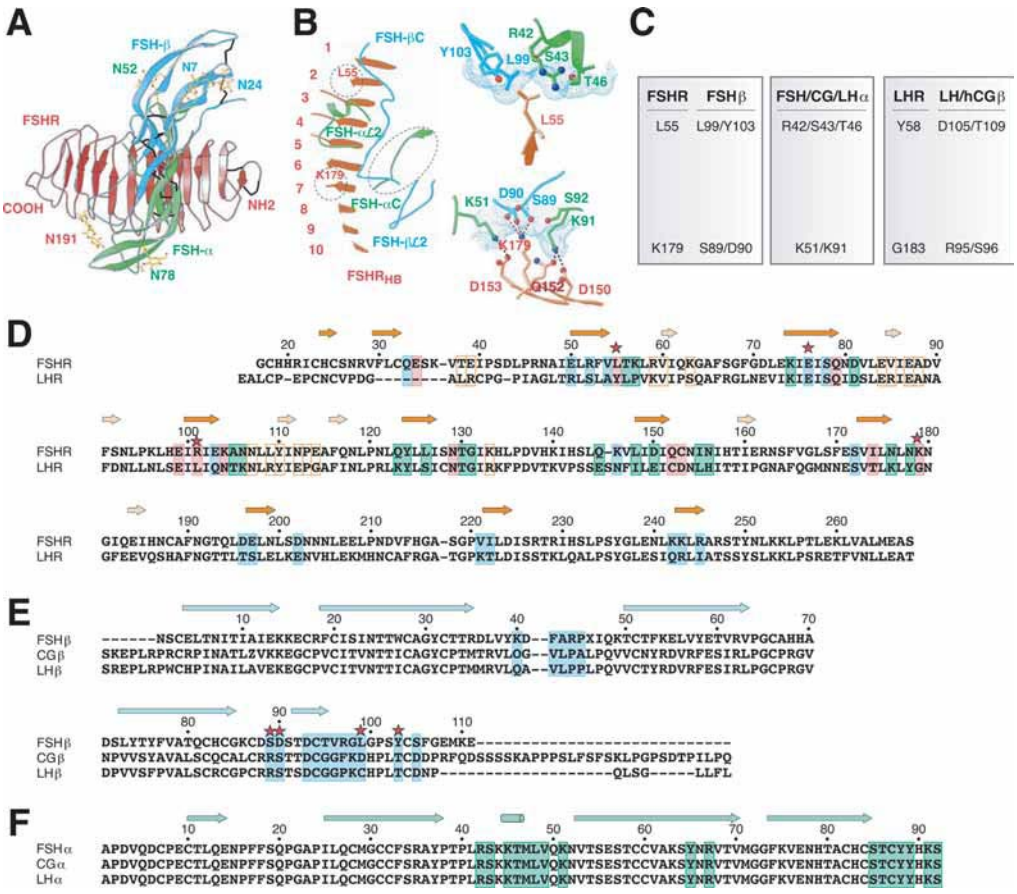


Fig. 2. Crystal structure of human FSH bound to FSHR$_{HB}$ *(45)*. **(A)** Ribbon diagram of the complex structure. Human FSHR extracellular hormone-binding domain (HB) in red. FSH-α and -β chain are in green and cyan, respectively. FSHR-β strand in thick red arrow correspond to 10 individual LRR. The observed N-linked carbohydrates at N52 and N78 of FSH-α, N7, and N24 of FSH-β and N91 of FSHR$_{HB}$ are in yellow. Disulfide bonds are in black. **(B)** Top view of the FSH–FSHR$_{HB}$ complex (left panel) regions of direct contact at the hormone/receptor interface. Dashed circles mark the locations of L55 and K179 in the FSHR$_{HB}$ structure. Detailed views of interaction (right panel). **(C)** Potential determinants of specificity, sequence variations indicate β-seat belt interaction with receptor residues at position 55 and 179 of FSR$_{HB}$ that determine specificity between FSH and TSH vs LH/hCG. The residues corresponding to LHR and human LH/hCG β are indicated. **(D)** Sequence alignment of human FSHR and LHR in the hormone-binding region of LRR. β-Strands located at the concave face of FSHR (red arrows) and in convex face (pink arrows). FSHR$_{HB}$ residues that interact with FSH-α alone in green, (FSHβ alone in cyan and both in magenta. Residues for dimer surface are boxed in orange. Residues implicated in binding specificity are marked by asterisks. **(E)** Sequence homology of β-subunit of human FSH and LH/hCG. The residues at the FSH/FSHR$_{HB}$ interface are highlighted in blue. **(F)** The common human α-chain sequence. The residues of FSH-α at the FSH/FSHR$_{HB}$ interface are highlighted in green. Panels C and F were added based on the information in ref. *45*. (Reproduced with permission from *Nature*.)

FSH-β loop is present at the interface. The interactions between receptor residues 55 and 179 and the hormone seatbelt and common α-subunit residues, determine the specificity between FSH/TSH and LH/hCG. The L^{55} residue of FSHR forms hydrophobic interactions with L^{99} and Y^{103} of FSH-β and with the aliphatic part of R^{42} in FSH-α. Substitution of L^{55} in FSHR with the larger tyrosine residue of the LHR (Y^{58}), which cannot be accommodated in the shallow pocket of L^{55}, significantly impairs FSHR-binding. Residues D^{105} and T^{109} of the LH or CG-β molecules, which correspond L^{99} and Y^{103} of FSH-β, could form polar interactions or hydrogen bonds with the Y^{58} of the LHR *(45)*. Another area of specificity resides with K^{179} of the FSHR, whose side chain is accommodated in a channel with sides bordered by residues K^{51} and K^{91} of FSH-α, which make universal salt bridges with two invariant acidic residues D^{150} and D^{153} in the FSHR. The top is covered by S^{89} and D^{90} of FSH-β, which form three hydrogen bonds contacts with K^{179} of the FSHR. These residues are involved in specificity, because of their substitution in FSH by the hCG or LH-β (Arg^{95}, Ser^{96}) counterpart permitted FSH to bind the LHR *(81)*. Furthermore, mutation of K^{179} to the Gly residue found in LHR (G^{183})

conveyed hCG-binding activity to the FSHR *(82)*. Another specificity site involving structurally adjacent FSHR residues E^{76} and $R^{101,}$ which interact with R^{97}, V^{101} of FSH-β is more concerned with FSH vs TSH selectivity *(45)*.

Models of receptor activation have included conformational changes in the ligand-binding induced by the hormone that are transmitted to the seven-TM module *(46)*; hormone-release inhibition with involvement of linker region *(83)*; and interaction of the bound hormone with binding site(s) in the seven-TM domain *(43,46,71,72,83)*. The FSH–FSHR structure is compatible with the proposed model of hCG-binding to the ribonuclease inhibitor *(72)*, which placed the α-(1,3) tip proximal to the TM/loop module within the TM domain. Signal transfer would be initiated by hormone contacts impacting the seven-TM barrel after displacement of the extracellular loop 2, and receptor dimerization could have a role in signal transduction *(45)*. In addition, the hinge region could participate because chimeric receptor studies have demonstrated that the interactions between the ectodomain and EL2 of the LHR are important in receptor constraint *(84)*. Furthermore, photoaffinity labeling and crosslinking experiments suggest a direct interaction between the hinge region and EL2 of the LHR *(85)*.

STRUCTURE–FUNCTION: MUTATIONS AND DISEASE

Major insights into the structure–function properties of the LHR have been derived from the expression of constructs containing engineered mutations, permutations, and deletions within its sequence, as well as naturally occurring point mutations that cause developmental and reproductive disorders. The several naturally occurring mutations identified in the human *LHR* gene cause either hormone-independent gain of male gonadal function because of activating mutations of the LHR, or loss of function as a result of inactivating mutations in both sexes.

The autosomal inherited condition of familial male-limited precocious puberty (FMPP), also known as testotoxicosis, results from activating mutations and is characterized by premature Leydig cell differentiation and hyperplasia, and early spermatogenesis. Such patients show signs of puberty from 1 to 4 yr of age, and their Leydig cells actively secrete testosterone with blood levels in the pubertal range, despite of their low or undetectable circulating LH and FSH. A similar phenotype is observed in sporadic cases of this disorder caused by somatic activating mutations that induce Leydig cell tumors and precocious puberty. All reported activating mutations to date are missense point mutations within exon 11 (Fig. 1; Table 1). Most of these mutations are located in the sixth TM domain and C-terminal region of the third intracellular loop, which is essentially a continuation of the sixth TM helix. However, predictions derived from models based on the structure of bovine-rhodopsin differ in the extent of the composition of IL3 *(86–88)* (Fig. 1). Mutations also occur in other TM helices, except TM4 and TM7.

Mutation of Asp^{578} to Gly is the most common cause of FMPP cases in the United States, where there appears to be a strong founder effect, and has not been identified in Europe *(89–91)*. The Ile^{542} Leu mutation in TM5 was found in four Dutch kindreds *(90,92)* and the Ala^{568}Val mutation is the most frequent cause of FMPP in Brazilian boys *(93)* whereas other mutations are more widely distributed. The Asp^{578}Gly mutation also occurs in some cases of sporadic hormone-independent sexual precocity. Mutation of Leu^{457} to Arg was found in a sporadic case of gonadotropin-independent precocious puberty *(94)*. In contrast to germline mutations of Asp^{578} to Gly, Tyr, and Glu, the Asp^{578} to His change is an active somatic mutation found in boys with Leydig cell tumor and precocious puberty who display signs of sexual development between age five and nine *(95,96)*. This is in contrast to the earlier occurrence of puberty in most patients with FMPP, typically before the age of four. However, boys with FMPP because of the Asp^{578} Tyr mutation, show an early onset of precocious puberty at one year of age, consistent with the strong activating nature of the substitution *(90,92,97)*. Also, a 20-mo-old infant with FMPP resulting from Asp^{578} Glu has been reported *(98)*. Another patient with FMPP developed a testicular seminoma at age 35 *(99)*, and a boy with the Asp^{564}Gly mutation was found to have testicular nodular Leydig cell hyperplasia *(100)*. The presence of tumors associated with somatic mutations and with FMPP has led to the proposal that Leydig cell activation might be a factor in the development of testicular tumors *(95,100)*. It has also led to the speculation that neoplastic transformation of Leydig cells involves inappropriate costimulation of the cAMP and phospholipase pathway *(95)*. These activating mutations do not engender a phenotype in females, most probably because of the minor expression of the LHR at prepubertal stages.

Several transfected cell lines, including COS-7, HEK, 293, and MA10 cells, expressing activating mutant receptors show high basal cAMP production and/or increases in cAMP-responsive reporter activity because of the constitutive activity of the expressed

Table 1
Human LHR Mutations

Location	*Amino acid*	*Nucleotide*	*References*
Activating mutations of LHR that causes disease[a]			
Missense mutations			
TM1/E11	$L^{368} \rightarrow P$	$CT^{1103}G \rightarrow CCG$	*93*
TM1/E11	$A^{373} \rightarrow V$	$GC^{1118}C \rightarrow GTC$	*193*
TM2/E11	$M^{398} \rightarrow T$	$AT^{1193}G \rightarrow ACG$	*97,101,194*
TM3/E11	$L^{457} \rightarrow R$	$CT^{1370}C \rightarrow CGC$	*94*
TM5/E11	$I^{542} \rightarrow L$	$A^{1624}TT \rightarrow CTT$	*90,92*
IL3/E11	$D^{564} \rightarrow G$	$GA^{1691}T \rightarrow GGT$	*90,92*
IL3/E11	$A^{568} \rightarrow V$	$GC^{1703}T \rightarrow GTT$	*93,103,195*
TM6/E11	$M^{571} \rightarrow I$	$ATG^{1713} \rightarrow ATA$	*104*
TM6/E11	$A^{572} \rightarrow V$	$GC^{1715}A \rightarrow GTA$	*105*
TM6/E11	$I^{575} \rightarrow L$	$A^{1723}TC \rightarrow CTC$	*92,102*
TM6/E11	$T^{577} \rightarrow I$	$AC^{1730}C \rightarrow ATC$	*104,196*
TM6/E11	$D^{578} \rightarrow H$	$G^{1732}AT \rightarrow CAT$	*89–92,95–98,197,198*
	$D^{578} \rightarrow Y$	$G^{1732}AT \rightarrow TAT$	
	$D^{578} \rightarrow G$	$GA^{1733}T \rightarrow GGT$	
	$D^{578} \rightarrow E$	$GAT^{1734} \rightarrow GAA$	
TM6/E11	$C^{581} \rightarrow R$	T^{1741} GC → CGC	*90*
Inactivating mutations of LHR that causes disease[b]			
Missense mutations			
EC/E4	$C^{131} \rightarrow R$	$T^{391}GT \rightarrow CGT$	*199*
EC/E5	$V^{144} \rightarrow F$	$G^{440}TC \rightarrow TTC$	*127*
EC/E7	$F^{194} \rightarrow V$	$T^{580}TC \rightarrow GTC$	*130*
EC/E11	$C^{343} \rightarrow S$	$T^{1027}GT \rightarrow AGT$	*125*
EC/E11	$G^{354} \rightarrow K$	$G^{1060}AA \rightarrow AAA$	*200*
TM4/E11	$L^{502} \rightarrow P$	$CT^{1505}C \rightarrow CCC$	*129*
TM5/E11	$C^{543} \rightarrow R$	$T^{1627}GT \rightarrow CGT$	*125*
TM6/E11	$A^{593} \rightarrow P$	$G^{1777}CC \rightarrow CCC$	*123,201*
TM7/E11	$S^{616} \rightarrow Y$	$TC^{1847}T \rightarrow TAT$	*121,124*
TM7/E11	$I^{625} \rightarrow K$	$AT^{1874}A \rightarrow AAA$	*187,202*
Nonsense mutations			
TM4/E11	$W^{491} \rightarrow X$	$TGG^{1473} \rightarrow TGA$	*202*
TM4/E11	$C^{545} \rightarrow X$	$TGC^{1635} \rightarrow TGA$	*107*
IL3/E11	$R^{554} \rightarrow X$	$C^{1660}GA \rightarrow TGA$	*124*
TM7/E11	$Y^{612} \rightarrow X$	$TAT^{1660} \rightarrow TAG$	*126*
Others			
EC/E1	^{18}LLKLLLLLQ(LQ) Insertion	^{55}CTGCTGAAGCTGCTGCTG CTGCTGCAG(CTGCAG)	*202,203*
TM6/E11	$A^{589} \rightarrow X^{605}$ Insertion/ frame shift	^{1741}GCC → TGCC	*119*
TM7/E11	^{608}LV deletion	^{1822}CTGGTT Deletion	*120*
E8 deletion			*121*
E10 deletion			*118,122,131*
Mutations of LHR with known effect			
Polymorphisms			
EC/E1	^{18}LQ Insertion	^{54}CTGCAG	*134,135,144,204*
EC/E4	$R^{124} \rightarrow Q$	$CG^{371}A \rightarrow CAA$	*202*
EC/E8	$L^{204} \rightarrow L$	$C^{610}TA \rightarrow TTA$	*202*
EC/E10	$N^{291} \rightarrow S$	$AA^{872}T \rightarrow AGT$	*121,134*
EC/E10	$N^{312} \rightarrow S$	$AA^{935}T \rightarrow AGT$	*134*
EC/E11	$D^{355} \rightarrow D$	$GAC^{1065} \rightarrow GAT$	*134*

[a]FMPP, Leydig cells hyperplasia.
[b]LC hypoplasia.
EC, extracellular domain; TM, transmembrane domain; Il, intracellular loop; E, exon; Δ, change; X, stop codon.

receptor *(16)*. Although, such mutant receptors activate Gs in a constitutive manner, furthermore, activation is elicited by agonist treatment in vivo and in vitro. In many cases, hCG stimulation of cells transfected with the mutant receptor elicits impaired second message responses *(90,98,101)*. In some instances the mutant-expressing cells including Cys^{581}, Arg, Ile^{542}, Leu *(90)*, and Leu^{457} Arg *(94)* are unresponsive to hormonal stimulation, and this is associated with loss of circulating testosterone responses to hCG. In the majority of cases there is no change in the affinity of such mutant receptors for the hormone *(102–105)*. In addition to activation of the Gs/adenylate cyclase/cAMP pathway, the somatic Asp^{578} His LHR mutant present in testicular tumors activates the PI pathway, when transfected in COS-7 cells, with increase of basal and hormone stimulated production of inositol phosphate *(95)*. Although, the mutant LHR is more highly expressed in the transfected cells than the wild-type receptors, stimulation of the PI pathway is observed also in presence of low levels of transfected receptors. Thus, it appears that the elevated constitutive activity is inherent to the mutant receptor rather than a result of overexpression. Furthermore, other mutants that cause strong constitutive activation of the cAMP pathway, including Asp^{578} to Leu/Tyr/Phe and Asp^{564} to Leu and three engineered double mutants of this of the Asp^{578} and Asp^{564} residues, when transfected into COS-7 cells exhibit minor to moderate agonist-independent inositol phosphate production when compared with cAMP production *(9,106)*.

Asp^{578} and other residues in TM6/IL3 are believed to maintain the receptor in an inactive state, through electrostatic or hydrogen bonding with residues in closely adjacent TM helices *(9,89)*. Hormonal stimulation and natural mutations cause conformational changes that lead to interaction with G protein(s) and initiation of intracellular signaling. The C-terminal portion of the IL3 *(103,107)* and other cytosolic interfaces could participate in G protein coupling. Different mechanisms of cAMP induction have been proposed by comparing constitutively activating LHR ($D^{578}G$) mutants and wild-type hCG-activated LHR stably expressed in HEK293 cells transiently transfected with C-terminal peptides of Gαs and Gαi2. The CP of Gαs can serve as competitive inhibitor of such interaction *(108)*, and decreases both basal and maximal cAMP stimulated by hCG in wild-type and mutant receptor. In contrast, cotransfection with GαiCP causes an increase in basal and hCG-induced cAMP production in cells expressing the mutant receptor but not the wild-type. These data indicate that the hCG-activated LHR assumes a conformation that differs from that of the mutant receptor, and implies allosteric interactions between Gαs and Gαi *(109)*.

The nature of the residues involved in mutant receptors is important, because replacement of Asp^{578} by an Asn with comparable size and charge *(110)* instead of Gly, Glu, Ser, Leu, Phe, or Tyr caused no change in receptor activity *(9)*. The Asp^{578} side chain in TM6, rather than its negative charge, is important for maintenance of the inactive state of the LHR *(9,98)*. In contrast, Asp^{564} to Ala, Val, Leu, Phe, or Asn mutations cause constitutive activation, but Glu do not, indicating a role of the negative charge in maintenance of the inactive state *(106)*. Moreover, replacements of Leu^{457} with positively charged side chains also cause constitutive activation *(111)*. Proposals based on modeling and mutagenesis have indicated the requirement of interactions between specific residues in TM3 and TM6, and of TM6 with TM7 in maintaining the inactive structure of the LHR. The formation of a salt bridge between the replaced R for Leu^{457} in TM3 and Asp^{578} in TM6, with reduction of constraint and increased solvent accessibility of relevant amino acids at the cytosolic interface between TM3 and TM6, participates in the constitutively active state *(112)*. Moreover, the wild-type Asn^{615} and Asn^{619} are required for the activity of $L^{457}R$. Conversely, disruption of hydrogen bonds of the hLHR Asp^{578} and Asn^{615} could induce the constitutive activity caused by mutations of D^{578} to G/L/H/F/Y. Other studies have indicated that the $L^{368}P$ mutation in TM1 could impact on a salt bridge interaction between D^{405} in TM2 and R^{464} in TM3 of the LHR *(93)*. In this case, the opening of a crevice between IL2 and IL3 could increase G protein accessibility and cause activation of receptor-mediated signal transduction. To date, no naturally occurring activating mutation has been found in the EC. It has been proposed that constitutive activation of the LHR might be associated in part with a conformational change of the EC and stabilization of the receptor in an active conformation.

Limited proteolysis studies have demonstrated that the wild-type receptor is more susceptible to enzymatic degradation than activating LHR mutants $A^{373}V$ (TM1), $L^{457}R$ (TM3), and $D^{578}Y$ (determined as the loss of hCG-binding capacity from transfected cells). These data suggested that exposure of the ectodomain to protease is decreased in the constitutively active hLHR because of its more tight conformation, and association with the helical bundle than in the inactive state *(113)*. It remains to be determined if such changes are also observed in the receptor when activated by the hormone. Other studies based on the constitutively active mutation of the TSH receptor at Ser^{281} revealed that mutation of the corresponding conserved neutral S^{277} of the LHR to selective nonpolar hydrophobic residues (L, M, I, V) in the cysteine-rich hinge region adjacent to the TM confers constitutive receptor activation, with increased basal cAMP production, and ligand affinity. In addition, replacement of the adjacent P^{276} by a flexible glycine causes receptor

activation comparable with the $S^{277}I$ mutation. This led to the proposal that the ectodomain of the LHR constrains the TM region, and that ligand-binding or point mutations on the hinge region could relax the interaction between the hinge region and EL2, resulting in conformational changes in the TM regions and Gs activation *(114)*.

Leydig cell hypoplasia (LCH) and aplasia resulting from several types of inactivating mutations in various parts of the *LHR* gene cause a rare form of male pseudohermaphroditism *(115–117)* (Fig. 1; Table 1), resulting from failure of fetal testicular Leydig cell function and/or subsequent impaired LH action at puberty *(118)*. Most of the LHR inactivating mutations reported are homozygous recessive, and only a few cases were caused by compound heterozygous mutations. The various forms of the disease result from missense point mutations occurring in the EC region, and TM helices TM4–TM7. Nonsense mutations with stop codons were identified in TMs TM4, TM5, TM6, and IL3. The remaining mutations include a 33 bp insertion in exon 1, a single nucleotide (T) insertion at nucleotide position 1741 bp of exon 11 which caused a frameshift and early translation stop ($A^{589} \rightarrow X^{605}$) *(119)*. Also, a 6 bp microdeletion (amino acid [aa] ^{608}LV) in TM7 *(120)*, and deletion of entire exon 8 *(121)* or exon 10 *(122)*.

The several gradations of severity of the condition, from the mild type II with preservation of male phenotype, to complete form male pseudohermaphroditism *(115)* or Type I, are related to the nature of the mutation and the resulting impairment of receptor expression and or function, which affects the degree of responsiveness to hCG and signal transduction. In general, the residual activity of the receptor in vitro is well-correlated with the severity of the patient's phenotype. In the most severe form of the condition, afflicted XY subjects have female external genitalia, absence of pubic hair, urethral and vaginal orifices, and inguinal or abdominal testes containing seminiferous tubules with normal *(123)* or immature *(124)* Sertoli cells, scarce immature germ cells, no Leydig cells, and no secondary male sexual characteristics. Blood testosterone levels are very low and are not increased by hCG treatment, whereas circulating LH levels are elevated and FSH levels are in the normal range. The absence of breast development in these patients reflects the lack of substrate for estrogen formation, (biosynthesis), and distinguishes this condition from testicular feminization as a result of mutations of the androgen receptor. Generally, this disease is reported in prepuberal, adolescent, and adult males. A single case of frameshift mutation at A^{589}, with early termination of translation 17 amino acids downstream, was recently reported in a 46XY newborn with complete female external genitalia and palpable testes *(119)*. Various degrees of milder phenotypes cover a range from micropenis to severe hypospadias and hypoplastic external genitalia. The presence of micropenis indicates that sufficient production of testosterone in fetal life occurs during the development of the external genitalia, followed by suboptimal production of androgen thereafter *(124)*. Female patients (46XX) with homozygous inactivating mutation of the LHR does not show abnormal female characteristics, but present with symptoms of primary amenorrhea and ovarian resistance to LH.

Functional analysis of inactivating mutant LHRs in transfected COS1/HEK293 cells reveals a marked to complete decrease in hCG-binding and hCG-stimulated cAMP production. The mutation/deletion or truncation might impair the ability of the receptor to adopt the active conformation for activation of signal transduction pathway. In other cases the mutation increases the intracellular accumulation of immature and/or misfolded receptors that are more susceptible to degradation and/or severe impairment of trafficking to the cell surface. The latter is responsible for reduced ligand-binding in most cases, as observed for the $C^{543}R$ mutant, $C^{343}S$ *(125)*, $Y^{612}X$ *(126)*, and other inactivating mutations. The $V^{144}F$ mutant lacks mature cell-surface receptor expression and is retained in the endoplasmic reticulum *(127)*. Based on the theoretical model of ligand-binding domain of glycoprotein hormone receptors *(72)*, the $V^{144}F$ mutation located in the convex side of the leucine rich repeats (LRR) might induce conformational strain in the molecule by overlapping interactions with F^{119}, whereas wild-type V^{144} does not. Steric hindrance might also cause abnormal processing and trafficking of the LHR protein, with consequent loss of function.

Retention of mutant receptors in the endoplasmic reticulum (ER) might result from the presence of misfolded and/or immature receptors that are recognized by specific ER chaperones. Distinct chaperones are associated with wild-type and mutant ($A^{593}P$, $S^{616}Y$) myc-tagged receptors stably transfected in HEK293 cells *(128)*. Calnexin and calreticulin (which assist the folding of proteins and prevent the exit of misfolded proteins from the ER) are associated with immature forms of both wild-type and mutant receptors. However, GRp94 and Bip (which prevent aggregation of newly synthesized proteins) are not associated with the wild-type LHR. Both Grp94 and Bip are associated

with the $A^{593}P$ mutant, but only Bip is associated with $S^{616}Y$ mutant. This finding suggests that the functional loss of activity of the mutants could result from the recognition of immature receptors with misfolded structure by specific ER chaperones, leading to decreased cell-surface expression. The $A^{593}P$ mutation in TM VI markedly reduces surface expression without affecting the K_d of the residual ligand-binding sites, and abolishes ligand-induced signal transduction *(128)*. Similarly, the $L^{608}V$ deletion in TM VII causes marked reduction in membrane expression, with preservation of high-affinity-binding and retention of receptors intracellularly. The marked reduction of cAMP activation by hCG is far below levels observed with comparably low numbers of wild-type receptors, indicating impaired signal transduction. However, receptor trafficking is not affected by the L^{502} P mutant in TM IV. In this case, as for the wild-type receptor, the mutant LHR is transported to the cell membrane in transfected cells, but displays minimal ligand-binding and consequently reduced cAMP stimulation by hCG in transfected cells *(129)*. Similarly, although truncation at the VII TM resulting from Y^{612} Stop causes loss of surface-binding and cAMP production, the mutant receptor shows normal cellular localization. This highlights the relevance of the TM VII and the C-terminal region in receptor binding and activation *(126)*.

The complete absence of exon 10, which causes Type II LCH, does not affect hCG action but LH action is impaired. Although, the binding affinities for LH and hCG are comparable to the wild-type, cAMP production is only significantly reduced in Δ exon-10 receptor stimulated by LH. This indicates that exon 10 of the LHR, which encodes for the hinge region, has no role in hormone-binding, but is relevant to receptor activation by LH. This exon is probably necessary to provide the residues to release the constraints of the receptor upon hormone-binding, and to facilitate the effective contact of the hormone required for receptor stabilization and activation. Thus, the functional nature of this mutation explains the presence of a male phenotype at birth, as a result of competent receptor activation by hCG during fetal life, and the retarded pre/pubertal development with small testicles and delayed bone maturation, with low testosterone levels owing to impaired Leydig cell stimulation by LH *(122)*. It is of interest that the LHR of the marmoset monkey lacks exon-10 of the *LHR* gene present in other mammalian systems *(131)*, and could thus be considered as the counterpart of the human mutation. However, the lack of an LCH (type II phenotype) in these species indicated that the marmoset gonadotropin competently activates the cognate receptor. Interestingly, CG is the only gonadotrophin with LH activity present in the marmoset pituitary *(132)*, and the carboxyterminal peptide present in CG but not in LH can overcome the absence of exon-10 and promote activation of the receptor *(133)*.

In addition to the activating and inactivating mutations described earlier, numerous single-nucleotide polymorphisms (SNPs) are present in the LHR. An LQ insertion at position 18 of exon-1, and two variable amino acids located in exon10, $N^{291}S$, and $N^{312}S$, are the most frequent LHR polymorphisms *(121,134)*. This is of interest in view of findings on the differential effects of LH and hCG in the absence of exon 10 *(118,122)*. Studies on an initial cohort of 266 breast cancer patients of Caucasian descent *(135)*, and more recently in 751 cancer patients with complete follow-up *(136)*, revealed that a genetic variant (LHR18insLQ) is associated with the age of diagnosis and prognosis of breast cancer. The LQ insertion with allele frequency in random population of 0.37 is located in the hydrophobic region of the signal peptide *(137)*. Although carriers were not found to have a higher risk for breast cancer, women who were homozygous for the LHR18ins LQ allele were 8.3 yr younger at diagnosis compared with those homozygous for the wild-type. In addition, patients who were LHR18insLQ carriers (heterozygous or homozygous) had a significantly worse overall survival, as well as nodal involvement and larger tumor size *(135)*. More recent in vitro studies have shown that the LRH18insLQ protein is translocated to the endoplasmic reticulum more efficiently. This results in a more sensitive mature LHR protein, associated with a higher level of expression and a significant increase in EC50, when compared with its non-LQ counterpart *(136)*. Further in-depth analyses will be required to determine whether the variant form of the receptor alters estrogen production by the ovary, displays intrinsic activity, or is impacted by LH or hCG in breast tissue to promote breast carcinogenesis.

The LHR *Gene*

The structure and function of the *LHR* gene has been elucidated in the rat, human, and mouse, and is highly conserved between species *(35–37)*. The *LHR* gene of over 70 kb is made up of 11 exons and 10 introns, all located within the region encoding for the EC of the receptor. Exons 1–10 encode for most of the EC and exon 11 for the rest of the receptor, including 47 aa of the EC adjacent to the plasma membrane, the TM region, connecting loops, and the cytoplasmic tail. Exon 11 also contains sequences of the 3′ flanking regions. Exon 1 contains the 5′ flanking region, the cleavable signal peptide of 26 aa in the pig *(32)*, rat *(31)*, mouse

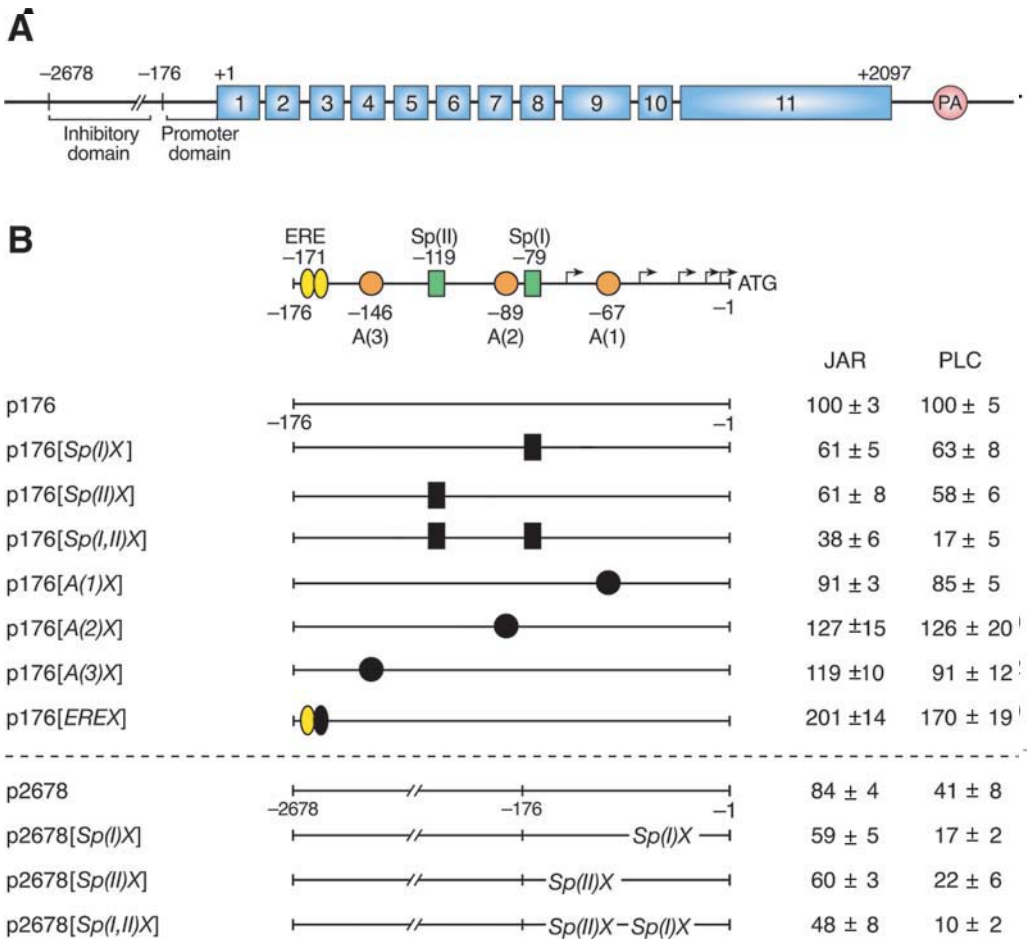


Fig. 3. Determination of the function domain in the promoter of *hLHR* gene. Transcriptional activity of wild-type and mutant constructs of hLHR promoter (–176 bp) in the presence or absence 5′ upstream sequences (–2678 bp) in JAR and SV40 transformed placental cells (PLC) *(145)*. **(A)** Schematic diagram of *hLHR* gene structure (exons 1–11) encoding the coding region (nt +1 as translational initiation codon and +2097 as termination codon) with 5′ 176 bp promoter and upstream inhibitory domain (–176 to –2678 bp). **(B)** Top panel: Schematic diagram of the 176 bp promoter with locations of activating Sp1/Sp3 elements (Sp[I], Sp [II]) (gray box), ERE half-site inhibitory (oval) and nonfunctional AP2 like elements (1–3) (circle), elements derived from mutation analysis. Arrow: transcriptional start sites. Transcriptional activity in wild-type p176 (–1 to –176 bp, middle panel) and p2678 constructs (–1 to –2678 bp, lower panel) and constructs with deletions (X). Filled black circle(s), oval, and square(s) represent mutant elements. PA, polyadenylation sites. (Please *see* color version of this figure in color insert following p. 180.)

(34), and human *(33)*, as well as four cystines. The general leucine-rich repeat motif of approx 20 amino acids is represented genomically by the regular insertion of introns at 70 bp intervals between exons 2 and 8. The LHR is encoded by a single copy gene, which has been mapped in the human to chromosome 2p21 *(138)*.

THE LHR THE 5′ FLANKING REGION AND PROMOTER STRUCTURE: FUNCTIONAL DOMAINS

The LHR Promoter

Studies on the structure and regulation of the *LHR* gene were initially focused on the rat *(7)*, and more recently information has been derived from studies on the regulation of the human gene. The *LHR* gene is TATA less and its GC-rich promoter resides 176 bp 5′ to the ATG codon in the rat and human receptors in gonadal and nongonadal tissues *(139–141)*. The major transcriptional sites are located within the promoter domain *(7,36,139,142–144)* and Initiator (Inr)-like elements encompass the transcriptional sites. LHR promoter activity is driven by two functional Sp1/Sp3-binding domains. The GC-rich Sp1 DNA-binding Sp1 domains and Inr elements in the LHR might operate as a Sp1/Inr-directed transcriptional complex. An ERE-half site domain that binds orphan receptors ER2 and ER3 (inhibitory) and testicular receptor 4 (TR4) (stimulatory) is located within the

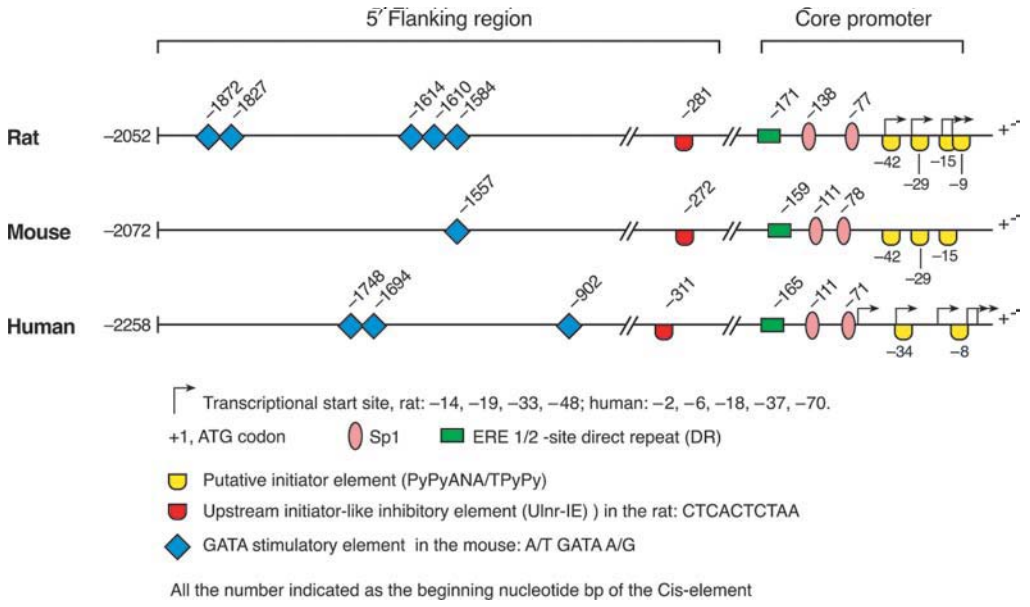


Fig. 4. Schematic representation of the location of functional *cis*-element in the core promoter and 5′ flanking region of rat, mouse, and human *LHR* gene. Putative initiator elements, activating Sp1 sites and inhibitory ERE half-site are aligned in the promoter core of rat, mouse, and human *LHR* gene *(36,38,145)*. Upstream initiator like element and GATA element were identified in the 5′ flanking region of the gene. Upstream initiator like element was demonstrated as a suppressor in the transcription of rat *LHR* gene *(149)*. By binding to GATA-4 transcriptional factor, GATA elements function as a transcriptional activating site in mouse adrenocortical tumorigenesis *(152)*. (Please *see* color version of this figure in color insert following p. 180.)

promoter upstream of the Sp1/Sp3 elements *(144,145)* (Fig. 3).

Upstream Inhibitory Sequences

In several cells examined, the rat *LHR* gene is constitutively inhibited by several sequences upstream to the promoter within –176/–2056 bp *(35,36,146–148)*. In contrast, only minor inhibition (15–20%) was caused by the presence of upstream 5′-sequences to the hLHR promoter domain in HeLa and JAR (human placental choriocarcinoma) cells *(35,146)*. However, a more prominent decrease in activity (by 60%) was observed in placental cell line (PLC) cells (normal human placental SV40 transformed cells) (Fig. 3). The differences observed between the human (minimally or less inhibition) and the rat (with nearly complete abolition of promoter activity) could be because of the presence of specific regulatory proteins related to sequences differences in the 5′ flanking region of the *LHR* gene between species. An upstream Inr-type element at –290/–282 bp that is conserved in the rat, mouse, and human *LHR* genes was identified as a suppressor domain in the most proximal inhibitory region (–482/–186 bp) 5′ to the rat LHR promoter *(149)*. This element, which interacted with TFII-I and an unidentified nuclear protein, effectively competed for TFII-I interaction with Inrs from the core promoter ang could be responsible for the inhibition induced by the –482/–186 domain.

A single GATA element is present at –1557 bp in the mouse, five in the rat and three in the human (Fig. 4), and the GATA-4 factor was shown to bind its cognate element in the mouse gene. GATA-4 has been detected in murine interstitial cells throughout fetal life and postnatal development and in Leydig cell tumors. This factor was shown to activate the *LHR* gene upon coexpression in steroidogenic and nonsteroidogenic cells *(150,151)*. Thus, GATA-4 could contribute to derepression/activation of the *LHR* gene through its cognate element(s) within upstream inhibitory domains 5′ to the promoter. Furthermore, GATA and LHR mRNA and protein expression precede the appearance of gonadotropin-responsive adrenal tumors in transgenic mice expressing the inhibin α-subunit promoter SV10 T-antigen transgene *(152)*.

Regulation of the LHR Promoter

In both the rat and human LHRs, promoter activity is driven by two functional Sp1/Sp3-binding domains that bind Sp1/Sp3 protein termed Sp1-2 and Sp1-4 in the rat, and the corresponding functional sites Sp1 (I) and Sp1 (II) in the human at –79 and –119 bp, respectively (Fig. 3; refs. *36,37,145,146*). These sites contribute similarly to basal promoter activity and are of central importance in transcription of the *LHR* gene *(36,145, 146)*. In contrast to the Sp1 (I) and Sp1 (II) in the human

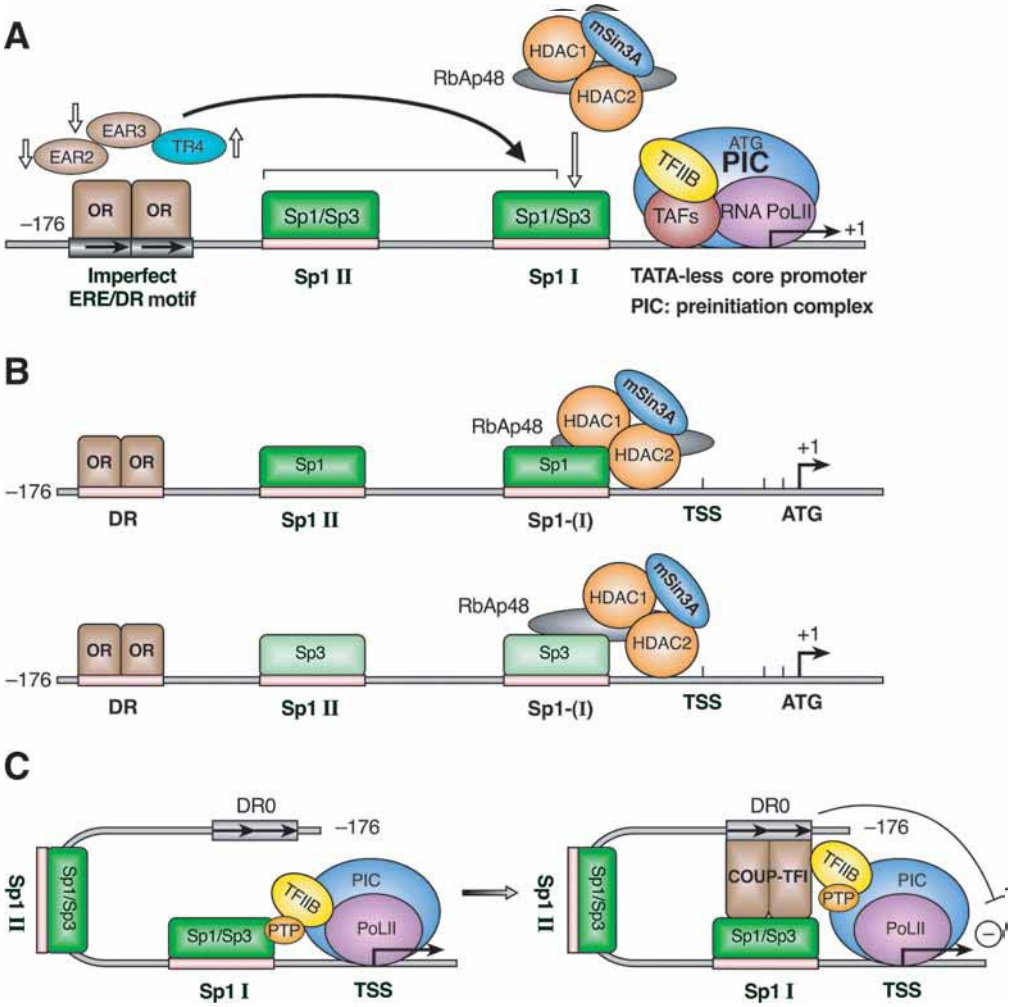


Fig. 5. Model of transcriptional regulation in the promoter domain of *hLHR* gene *(153–157,159)*. **(A)** 176 bp promoter with its functional domains, associated transcription factors, and silencing regulatory complex (HDAC/mSin3A/RbAp48). Orphan receptor: EAR2, ER3, TR4 arrows up/down = stimulation/inhibition. Sp1 I, Sp1 II: Sp1 sites that bind Sp1/Sp3 transcription factors. **(B)** Model of recruitment of HDACs/mSin3A corepressor complex to the *hLHR* gene promoter by Sp1 (direct, top) or Sp3 (indirect, lower). OR: orphan receptor. DR: ERE half-site direct repeat. Transcription start sites indicated by vertical bars. mSin3A, inhibitory member of the complex. RbAP48: HDAC associated protein. Recruitment of HDACs/mSin3A corepressor complex to the *hLHR* gene promoter by Sp1 through direct interaction with HDACs, and through additional link, RbAP48. The mSin3A is attached to Sp1 within the promoter through its interaction with HDACs. Indirect recruitment of HDACs/mSin3A to the promoter by Sp3 is mediated through RbAp48 which interacts with Sp3 and HDAC. **(C)** Model for active silencing of target gene expression by COUP-TFs. Repression of *hLHR* gene expression by COUP-TF1/EAR3 from crosstalk among Sp1/Sp3, COUP-TF1/EARs and TFIIB. TFIIB interacts with Sp1 indirectly through PTP and activate transcription. PoL II is recruited to the promoter. EAR2/EAR3 disrupt the interaction of TFIIB with Sp1/Sp3 and inhibit transcription. PTP, putative tethering protein; PIC; preinitiation complex; TSS, transcription start sites; ERE, estrogen responsive element; DR; direct repeat; OR, orphan receptor; HDAC, histone deacetylase. (Please *see* color version of this figure in color insert following p. 180.)

(145), the Sp1-4 domain in the rat is more complex and contains two overlapping nonidentical Sp1 sites. Only the 5′ element binds to Sp1/Sp3 protein, whereas the 3′ Sp1 like domain binds unidentified protein(s) that can sustain the Sp1-4 activity when the 5′ is mutated *(140)*. Upstream of the two functional Sp1 sites/domains, an imperfect estrogen receptor direct repeat with no spacing is present in the rat and human genes, respectively *(36,146,153,154)*. In addition, the promoter contains three GC-rich AP-2 like elements that are silent in the *hLHR* gene. The nuclear receptor orphan receptors EAR2 and EAR3/COUP-TF1 inhibit hLHR transcription through their interactions with the DR motif, and TR4 acts as an activator through the same response element (Fig. 5A). These orphans bind competitively and with high affinity to this motif and

exhibit differential-binding to the rat and human LHR promoters *(153,154)*. EAR3/COUP-TFI bind with threefold higher affinity to the human promoter than the rat promoter, as a result of the lack in the rat of a G 3′ to the second DR site and TR4 is not stimulatory in the rat owing to a single base-pair mismatch in the second DR site that abolishes its binding *(154)*.

Changes in endogenous levels of EAR2 and EAR3 during gonadotropin stimulation of rat granulosa cells correlate with derepression of promoter activity *(154)*. These orphans operate in repressive /inductive states of the rat LHR in granulosa cells of early follicles (repressive), but not in those of mid- to late-ovulatory follicles and luteal follicles, which are relieved of repression by the reduction of expressed orphan inhibitors induced by gonadotropins *(154)*. The human hDR binds EAR2, EAR3/COUP-TFI, and TR4 readily in the human testis and ovary, indicating opposing physiological regulation of *hLHR* gene transcription by these orphans in the human. These findings are relevant to the control of the hLHR by EAR2 and EAR3 during the menstrual cycle and to the regulation of the gene in Leydig cells *(153)*. The proximal Sp1-I-binding site is critical for the EAR2, EAR3/COUP-TFI repression in both human and rat, and its mutation reduces inhibition by EAR2 and abolishes inhibition by EAR3/COUP-TFI *(155)*. Cotransfection analyses in SL2 cells, which lack Sp1/Sp3, showed that both Sp1 and Sp3 are required for repression *(155)*. Mutual recruitment of EAR3 and Sp1/Sp3 bound to their cognate site furthermore supported functional cooperation between Sp1 and DR domains. Furthermore, EAR3 specifically reduced association of TFIIB with the Sp1-I site without interfering with EAR3′s interaction with the core promoter. TFIIB is a critical component in the regulatory control of EAR3 and Sp1/Sp3 on the initiation complex, and the perturbation of its association with Sp1 by EAR3, independent of histone deacetylase (HDAC) was reflected in reduced recruitment of RNA Pol II to the promoter and impaired LHR transcription *(155)*. Such cross-talk among EAR3, TFIIB, and Sp1/Sp3 reveals that repression of *hLHR* gene transcription by nuclear orphan receptors is achieved through perturbation of the communication between Sp1/Sp3 at the Sp1-1 site and the basal transcription initiator complex (Fig. 5C; refs. *155,156*).

Other studies have demonstrated that the *hLHR* gene is subject to epigenetic regulation, whereby local chromatin changes at the *LHR* gene promoter are critical for gene transcription. Inhibition of HDAC by trichostatin A (TSA) caused 40-fold gene selective induction of hLRH activity, indicating inhibition of potent constituitive repression of *hLHR* gene transcription *(157)*. TSA triggered recruitment of acetylated H3 and H4 to the hLHR promoter, which in turn induced changes in the chromatin environment, recruitment of Pol II and increases in gene transcription. The Sp1 (I) site was identified to be critical for the TSA effect. However, the mechanism of silencing of *hLHR* gene expression through alteration of HDAC activities is independent of the pathway involving unliganded orphan receptors. A multiple protein complex was found to associate specifically with Sp1 (I) site (Fig. 5B). This complex, in addition of Sp1/Sp3 bound to its element, is made up of HDAC1 and HDAC2 which interact directly with Sp1 and indirectly with Sp3 through the HDAC-associated protein Rbp48, and mSin3A is attached to the promoter through its interaction with HDACs. HDAC1 and HDAC2 were identified as repressors for the hLHR and mSin3A as corepressor in functional studies *(157)*. The regulated derepression of such inhibitory control of the *hLHR* gene, through as yet unidentified signal inputs, is of major relevance in the functional control of induction of *LHR* gene expression during differentiation, growth, and development of gonadal cells.

More recent studies have demonstrated that histone modifications have a dominant role in the control of silencing or activation of *LHR* gene expression. Site-specific lysine acetylation of histone H3 at K9, K14, and H4 at K12 is associated with *LHR* gene activation. In contrast, methylation of H3 at K9 is present at the silenced LHR promoter, respectively. Acetylation and deacetylation of histones H3/H4 have been shown to induce a relaxed and competent or a condensed and inactive chromatin, respectively *(158)*. Although DNA methylation levels do not affect the histone code of the *LHR* gene promoter, demethylation of the promoter CpG sites is necessary for maximal stimulation of this gene. DNA methylation and demethylation of the LHR are operative under the architecture set by histone modification. Alteration of the chromatin structure and concurrent DNA demethylation of the promoter CpG sites are required for release of the HDAC/Sin3A inhibitory complex, and possibly of other inhibitor(s) for derepression of the LHR promoter and maximal stimulation of transcription *(159)*.

LH RECEPTOR mRNA FORMS

Alternate Splicing

The diversity of RNA transcripts of the LHR identified in target organs and cells cultures is provided by alternate splicing of introns and the use of two polyadenylation domains. Several alternate truncated forms of the LHR lacking either the TM/cytoplasmic

Table 2
LHR Variants Resulting From Alternative Splicing

Name from literature	*Classification based on alternative splicing pattern*	*Source*	*Amino acids no.*[a,b]	*References*
Rat LHR				
rLHR2100	rLHR (FL)	Testis, ovary	700 (m)	*32*
B, B4, rLHR1834	Δ11	Testis, ovary	342[a] (s)	*35,205–207*
rLHR1759	–E5Δ11	Ovary	317[a] (s)	*207*
EB, rLHREB	–E9Δ11	Ovary	280[a] (s)	*205*
B3, rLHRB3	Δ10	Ovary	367[a] (s)	*206*
B1, rLHRB1	–E9Δ10	Ovary	305[a] (s)	*206*
B2, rLHR2075	Δ9	Ovary	251[a] (s)	*206,207*
C1, rLHRC1	In9	Ovary	294[b] (s)	*206*
C2, rLHRC2	In6	Ovary	183[b] (s)	*206*
rLHR1950	–E3/4	Ovary	650 (m)	*207*
A2, E, E/A2, rLHRE/A2	–E9	Ovary	638 (m)	*205,206*
Pig LHR				
A	pLHR (FL)	Testis	696 (m)	*31*
B	Δ11b	Testis	329[a] (m)	*31*
C	Δ11c	Testis	331[a] (s)	*31*
D	Δ11d	Testis	384[a] (s)	*31*
Human LHR				
	hLHR	Ovary	699 (m)	*33*
	–E9	Ovary	637 (m)	*161*
	–E9Δ11	Ovary	267[a] (s)	*161*
	Δ11	Ovary	329[a] (s)	*161*

[a]Premature stop codon.
[b]Stop codon in intron.
–E, entire exon deletion; E no., Δ no., or In no., exon or intron number; Δ, alternative internal 3′ acceptor mode; In, partial intron sequence insertion. FL, full length; m, membrane form; s, soluble form.

domain or exclusively the TM domain have been identified in rat and pig testis and human ovarian libraries. These conform to deletions of complete exons 3, 4, 5, and 9 mostly in the rat and human ovary, or partial exon regions (exon 11) predominantly in the rat and pig testis and also in the human ovary (Table 2). Deletion of exon 8 was observed in a patient with LCH *(121)*. The human ovarian splice variant lacking exon 9 was found to reduce the density of wild-type LHR expression by interaction with the immature 64 kDa of the receptor in transiently and stably transfected 293 cells with wild-type and splice variant constructs *(160)*. Complete deletion of exon 10 (homozygous) of the LHR was present in a patient with male hypogonadism in whom differential actions of hCG and LH were observed *(122)* (*see also* "Structure–Function: Mutations and Disease"). However, the transcript corresponding to this deletion was not observed in the human ovary (corpora lutea and luteinized granulosa cells) *(161)*. In addition, insertions within introns have been reported. Most of these forms yield truncated species with frameshifts and premature stop codons (Table 2). Also, several variants have been observed in SV40 transformed human placental and JAR choriocarcinoma cell lines. The existence of these forms in target tissues can only be inferred from the presence of truncated mRNAs and from expression studies in mammalian cells, with identification of the truncated soluble product in the medium of cultures or entrapped within the cells.

Polyadenylation Domains

The alternative use of polyadenylation domains, contributes to the mRNA differences observed, because the mRNAs of all the above forms could be influenced by different 3′ extensions. In the rat, the 6.2 kb 3′ noncoding region contains two functional domains, H1 (nt. 2368–2491) and H2 (nt. 5579–5768), each containing two distinct fuctional pA core signals (AAUAUA, A1 and A2 in H1 and AAUAA, A3, and A4 in H2) (Fig. 6). These generate two sets of mRNA species of 2.3 or 2.6

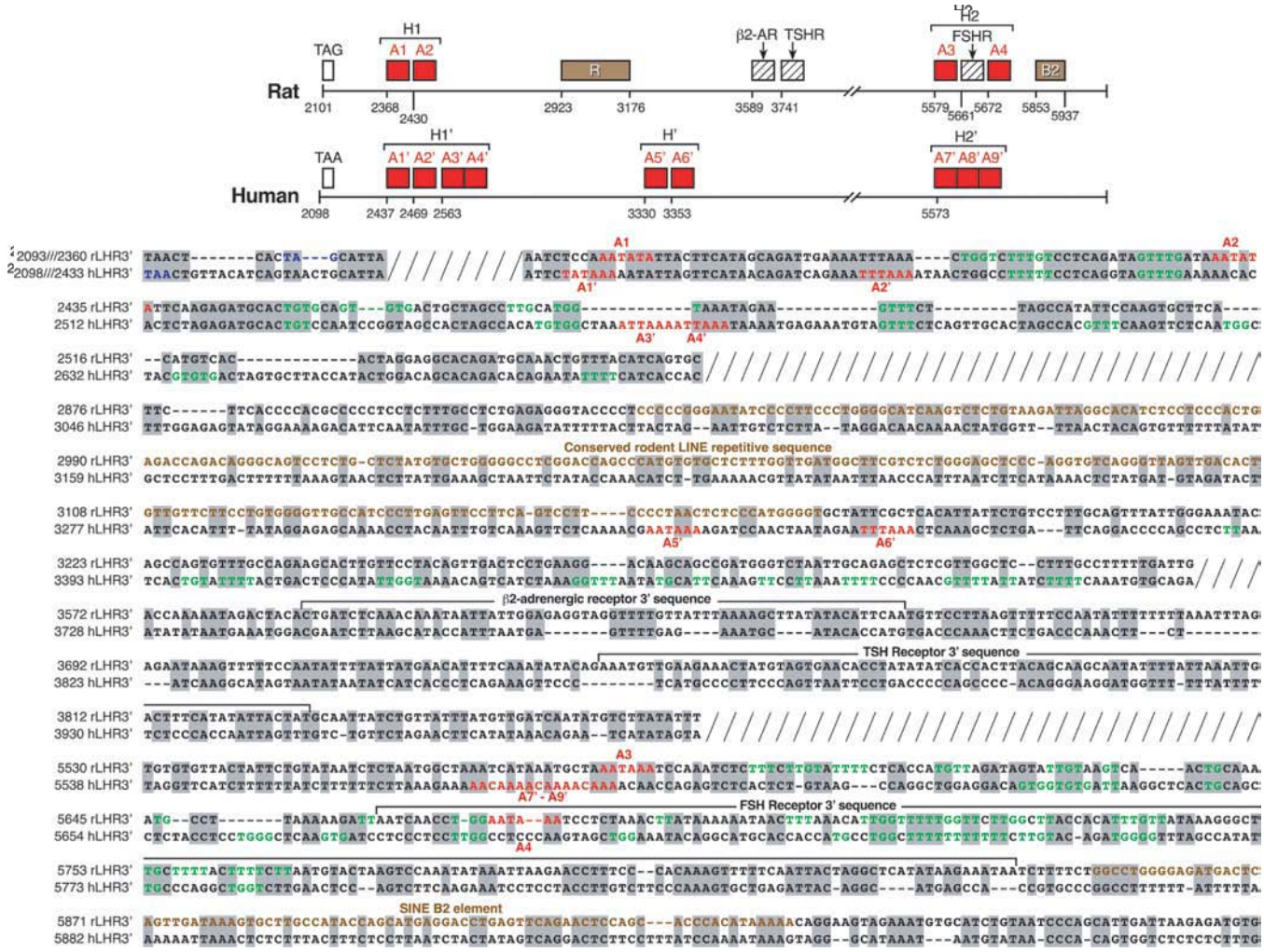


Fig. 6. Comparison of 3′ flanking sequences of the rat and human *LHR* genes. 3′ flanking comparison of rat and human LHR (rat, acc. No. NM_012978 and human acc. No. NM_000233/NC_000002) in schematic (top) and sequences (below) presentation begins with the termination codon of the coding region (rat-r, TAG at nt: +2101 and human-h, TAA at nt +2098) (dashed box). H# or H′#: Core polyadenlyation domain (black). A# or A′#: microheterogeneous poly (A) addition. H1 and H2 were functional polyadenylation domains in rat LHR *(15,166)*. Human polyadenlyation signals (H′#) were derived from databases of sequences independent of consensus AAUAAA signal *(208)*. Several diffused GT and T-rich sequences downstream of the hexanucleotides are present. Conserved rodent repetitive LINE sequences (R) (open arrowhead) and SINE B2 element (B2) (closed arrowhead). Homology region of LHR with rat β-adrenergic (β2-AR), and other glycoprotein hormone receptors (TSHR, FSHR) are marked as overhead region. ////: missing nucleotides. (Please *see* color version of this figure in color insert following p. 180.)

and 5.8 kb (heterogenous), respectively. The A3/A4 is three times more efficient than A1/A2 variant, because of the intrinsic pA signal sequence, distal elements (SINE B3 element, 3′ A3/A4, and repetitive LINE element 3′ of A1/A2) and tissue specific factors. In addition, AT-rich regions are found within or adjacent to the H1 and H2 domains. The presence of multiple copies of AU-rich motifs in the LHR could provide determinants for the control of degradation *(162)*. However, their selective removal from the LHR 3′ untranslated region had no apparent effect on mRNA stability or receptor expression *(163)*. There is an absence of sequence similarity to H1 in the TSHR, FSHR, and β-adrenergic receptors, indicating that this weaker domain was introduced later in evolution.

Alignment of the 3′ noncoding ends of the human and rat LHRs shows a region of significant similarity at H1 (71% identity). In the human, three putative pA domains variants (A1′–A3′) are present in the area corresponding to the H1 region of the rat LHR and are predicted to yield 2.4 kb mRNA species. In addition, AAUAAA (nucleotide [nt] 3330) (A3′) and UUUAAA (nt 3353) (A′) could be responsible for the processing of the 3.6 kb mRNA species. Furthermore, three consecutive overlapping AACAA signals (nt 5573) in the human corresponded to the H2 region in the rat (A7′, A8′, A9′).

Deletion studies in the rat revealed that the distal 1.98 kb region of 3.5 kb of the 3′ untranslated region confers an inhibitory effect on LHR or luciferase reporter gene expression in 293-T-cells *(163)*. Also, a potential *trans*-acting factor that binds to a polypyrimidine-reach bipartite sequence in the coding region of the LHR mRNA termed LRBP, and identified to be mevalonate kinase *(164)*, has been suggested to regulate rat mRNA LHR receptor stability and to contribute to gonadotropin-induced receptor down-regulation in the rat ovary *(165)*.

LHR mRNA Forms

Among the major mRNA forms present in the rat ovary and testis, the 2.3 and 2.6 forms correspond to the extracellular B form (alternative spliced form –Δ11, soluble) *(31,35)* and the holoreceptor, respectively. Also, the 5.8 kb species corresponds to the extracellular form B as well as the holoreceptor. The differences in the long and short mRNA species are attributable to extension of the 3′ noncoding region. The predominant 5.8 kb mRNA species processed by A_2 is made up of transcripts for the B form and the holoreceptor (form A). These are not discernable in Northern blots, but are revealed by polymerase chain reaction and each bears a 3′ extension of about 3.5 kb.

Multiple LHR mRNA species have been detected in the ovaries and testes of individual species *(31,35)*. In the rat ovary, three major LHR mRNA species of 5.8 kb, 2.3, and 2.6 kb and a minor band of 4.4 kb are present at most stages of ovarian maturation, and additional minor species are found at specific developmental stages *(166)*. In the rat Leydig cell, LHR mRNA species of sizes comparable with those of rat ovarian species were detected, but in lower abundance *(167)*. Three species of 6.7 kb (predominant), 2.6, and 2.3 kb were observed in mesenchimal progenitors cells and in immature and adult Leydig cells. Low, but detectable mRNA levels are present in progenitor Leydig cells and increase fivefold in immature Leydig cells (21–35 d postnatally), whereas only double those of the progenitor in the adult Leydig cell. This contrasts with the twofold and 15- to 20-fold increase in receptor-binding capacity over those of progenitor cells observed in immature and adult Leydig cells, respectively, indicating that differences in translational efficiency and/or maturational process of receptors at the various stages of development of Leydig cells modulates the number of functional receptors *(61,167)*. In the testis, transcripts of all sizes were observed at all ages (1.8, 2.7, 4.2, and 6.8 kb), indicating the combined utilization of pA domains and alternative splicing mechanisms. In the fetal testis, polymerase chain reaction analysis demonstrated the presence of truncated extracellular form B at day 14.5, 1 d before the appearance of the full length receptor transcript at fetal day 15.5 *(168)* and concomitant with detection of ligand-binding and LH induced stimulation of second messengers and androgen production. The 6.8 kb species was predominant in the testis of all ages and in the adult testis the 1.8 kb transcript (truncated form B) was also abundant *(168)*. In the human, testis and thyroid, 4.5 and 2.6 kb mRNA species were detected *(169)*, and three LHR transcripts of 5.4, 3.6, and 2.4 kb were found in the human ovary *(160)*. The sizes of these transcripts indicate the utilization of the deduced human pA core regions of LHR 3' RNA (*see* "Polyadenylation Domain Section", and Fig. 6).

Homologous downregulation of the LHR in vivo during follicular and luteal desensitization is correlated with the steady-state levels. The three major species (5.8, 2.6, and 2.3 kb) were present throughout differentiation and downregulation and changed similarly, the 5.8 kb species being consistently more abundant than the smaller forms. The expression of LHR during follicular maturation, ovulation, and desensitization is related to the prevailing levels of receptor mRNA in the ovary *(166,170)*. In contrast, in the Leydig cell in vivo desensitization of adult male rats with hCG caused dissimilar decrease of mRNA species, with more profound decreases of the longer forms and no change in a 1.8 kb species *(171)*. In the luteinized rat ovary *(172,173)* and cultured porcine luteal cells *(174)*, downregulation by the homologous hormone is related to a decrease in mRNA stability, whereas in mouse tumor Leydig cells, MA10 it is associated with decreased transcriptional activity *(175)*. It remains to be determined whether this is the case for the rat Leydig cell during in vivo desensitization by gonadotropin.

LHR Expression in Nontarget Tissues

In addition to its association with gonadal tissues, LHR messages and its expression products, have been demonstrated in a variety of nontarget human tissues *(176–180)*. Multiple LHR transcripts and translation products were localized in epithelial cells or the rat prostate (ventral lobe and peripheral tissues) *(181,182)*. hCG-binding to 80 kDa LHR protein and the stimulation of cAMP production by gonadotropin indicated that LH might regulate the function of this gland. Adrenal glands from several species contain LHRs *(181,182)*, and in human fetal adrenals hCG causes an increase on dehydropiandrosterone sulfate secretion *(183)*. Although the functionality of the LHR in adult

human adrenal is not known, in some conditions where these receptors might be operative (chronic anovulation, adrenarche, pregnancy) adrenal androgen production diverges from cortisol secretion. This may also apply to some cases of Cushing syndrome because of LH/hCG-sensitive adrenal tumors during pregnancy and menopause *(184–186)*. It has been postulated that these could arise from changes of sensitivity and/or zonal distribution of normal adrenal LHR receptors *(187)*. Recent studies have demonstrated that hCG treatment of human (H295R) adrenal cortical carcinoma cells, which have some characteristics of normal adrenal cells, stimulates the cAMP/PKA signal transduction pathway and increases in dehydroepiandrosterone sulfate mRNA and enzyme protein levels *(188)*. Other studies have indicated that transcription factor GATA-4 and the LHR, which are coordinately upregulated before the appearance of discernable adrenal tumors induced in mice by the inhibin α-subunit promoter/virus 40T antigen transgene, have a role in adrenal tumorogenesis *(152)*.

LHRs are expressed in epithelial cells in normal female breast, benign breast lesions, breast carcinoma, and breast cancer cell lines *(189,190)* and in normal male breast, benign gynecomastia and male breast carcinoma. LH/hCG has an antiproliferative and apoptotic effect on human breast epithelial cultures, and decreases the expression of estrogen receptors *(191)*. In contrast, other studies have demonstrated that hCG stimulates MCF-7 cell growth *(192)*. Recent studies have shown minor expression of LHR mRNA in MCF-7 (breast cancer) and JAR cells (choriocarcinoma) cell lines, and abundant expression in PLC cells (SV40 transformed normal PLC) *(159)*. However, LHR mRNA expression in JAR cells was induced by 40-fold in JAR cells by the HDAC inhibitor TSA, which caused acetylation of the promoter. Further increases up to 130-fold were observed upon demethylation of the promoter by the DNA methylation inhibitor AZAC. Moreover, in MCF7 cells where the promoter is basally unmethylated, TSA induced up to 130-fold increases in LHR mRNA expression. The maximal levels of expression were comparable with those observed in the unrepressed normal PLC *(159)*. These findings have indicated a requirement for coordinated changes in DNA methylation and histone modification of the LHR promoter region for regulation of LHR transcripts (*see also* "Regulation of the Promoter" section). They also suggest that breast cancer cells have high potential for derepression and elevated expression of receptors.

FUTURE PROSPECTIVES

Based on the FSHR crystallographic findings, it is likely that considerable structural development will be reported for the LHR. At present it is difficult to place the FSHR dimer findings in the context of the holoreceptor, as the interaction between the two receptors, each binding one molecule of hormone, leaves the TM regions far apart for association. In this regard it is not known whether the dimer is constitutively formed, or induced and the role of the dimeric form in signal transduction has yet to be defined. Further exploration is needed to identify the molecular interactions of the receptor that are responsible for initiating signal transduction. Also, more investigations are needed to determine the specific signal transduction mechanisms involved in individual cell types under various physiological conditions. The information expected to be derived from the LHR-null model about the role of extragonadal LHRs has not eventuated, and other avenues will need exploration to determine their relevance. The tumorigenic potential of gonadotropins and mechanism(s) involved therein, also, require further evaluation. Extension of the investigation and polymorphism of steroid-dependent neoplasms or other diseases could provide valuable information about these matters. The impact of such polymorphisms on cancer risk could be explored by the generation of mouse models of LHR variants. Evaluation of the molecular mechanisms induced by hormones, growth factors, and activators in LHR transcription and translation in vitro and in vivo settings should provide relevant physiological information about the control of LHR expression. Although a great deal of information is currently available on the mechanisms of repression/and derepression of the LHR transcription, and several components of activator/inhibitor complexes, and chromatin status have been characterized, additional key participants remain to be elucidated.

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17 Insights Into Leydig Cell Function From Mice With Genetically Modified Gonadotropin Action

Ilpo Huhtaniemi, MD, PhD

SUMMARY

The currently available molecular biological methods allow us to produce mouse models with gain- and loss-of-function mutations of specific genes. These techniques have been extensively applied to the study of hormones and receptors involved in the endocrine regulation of testicular function. There are now knockout mouse models for the gonadotropin subunits and receptors as well as transgenic models overexpressing the gonadotropin subunits. The models have partly confirmed and extended earlier knowledge and partly brought totally new information about the physiology and pathophysiology of gonadotropin action. The purpose of this chapter is to review the novel information about the effects of gonadotropins on Leydig cell function which has been obtained from genetically modified mice.

Key Words: Follicle-stimulating hormone; follicle-stimulating hormone receptor; gonadotropin action; human chorionic gonadotropin; knockout mice; luteinizing hormone; luteinizing hormone receptor; transgenic mice.

INTRODUCTION

The knowledge about gonadotropin function has been highly enhanced recently by the identification of human patients with inactivating mutations in gonadotropin subunit genes and activating and inactivating mutations in the cognate receptors (R). This novel information has been supplemented by observations on genetically modified mice with gain-of-function mutation (transgenic overexpression) of the gonadotropin subunit genes and loss-of-function mutations (knockouts) of the gonadotropin subunit and receptor genes. Altogether the clinical observations and novel experimental models have confirmed earlier concepts about gonadotropin functions during sexual development and maturity. In addition, they have shed light on novel and in many cases unexpected functions of these hormones, especially, at the extreme high and low ends of their dose–responses. Detailed reviews about the human mutations in gondotropin and gonadotropin receptor genes have been presented elsewhere *(1,2)*. This chapter concentrates on the information brought by the genetically modified mice about the role of the two gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH), in the regulation of Leydig cell function.

GENETIC MODIFICATIONS OF LH FUNCTION

LH provides the main tropic stimulus of Leydig cell differentiation, growth and steroidogenesis, and its crucial role in androgen production and consequent masculinization has recently been demonstrated in humans by inactivating mutations detected in the *LH-β subunit (3,4)* and *LHR (5,6)*. Normally men with the ligand mutation are masculinized at birth, because placental choriongonadotropin (hCG), an agonist of pituitary LH, is able to stimulate fetal Leydig cells. However, these men totally lack sexual maturation at puberty, because their Leydig cells remain undifferentiated and hypoplastic in the absence of LH stimulation. A stronger phenotype of pseudohermaphroditism is brought about by completely inactivating *LHR* mutations; these individuals almost completely lack the fetal masculinization of their genital structures, because the fetal Leydig cells are unresponsive to LH and hCG stimulation.

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Inactivation of LH Action

Common α-Subunit Knockout Mice

The first mouse model reported with genetically disrupted LH production was the glycoprotein hormone *common α-subunit* (*Cα*) knockout mouse *(7)*. Because of missing Cα the mice are unable to produce bioactive dimeric LH, FSH, and thyroid-stimulating hormone and are therefore, postnatally both hypogondal and hypothyroid. Nevertheless, the masculinization of (–/–) mice is normal at birth, providing further evidence that in the mouse, fetal Leydig cell differentiation and endocrine function are not dependent on LH action (discussed in more detail in following Subheading).

LH-β Knockout Mice

Targeted disruption of the *LH*-β was reported very recently *(8)*. The male phenotype of these mice is nearly identical with the previously reported knockout of *LHR*, and will be described in more detail in connection with the latter mutation which has so far been more extensively studied (*see* following Subheading). Mutant *LH*-β-null males were normally masculinized at birth, but showed no sexual maturation at puberty, had decreased testis size, prominent Leydig cell hypoplasia, defects in the expression of genes encoding steroidogenic enzymes, and reduced testosterone levels.

Interestingly, their androstenedione levels were increased. Their spermatogenesis was blocked at the round spermatid stage, in keeping with the finding in some other androgen deficient conditions with normal FSH action *(9,10)*. When the secretion of both gonadotropins is blocked, spermatogenesis stops before the second meiotic division *(7,11)*. The mechanism for this difference can be twofold; either FSH stimulates directly spermatogenesis, or it occurs because of increased paracrine stimulation of Leydig cell testosterone production, which can be demonstrated with FSH when gonadotropin secretion is very low (*see* following Subheading). No defects in FSH function were found in the *LH*-β knockout mice, and their reproductive function could be rescued by treatment with hCG. Because this model is remarkably similar to the *LHR* knockout mouse (*see* following Subheading), crosstalk of LHR in vivo with other structurally similar ligand–receptor pairs is unlikely.

LHR Knockout Mice

Two laboratories reported *LHR* knockout models in 2001. One of the mode was created by targeted deletion of the proximal part of the *LHR* promoter region and exon 1 *(12)*. In the other model *(13)*, LHR knockout was produced by targeted disruption of the long 11th exon of the receptor gene, encoding the transmembrane and intracellular domains of the receptor. Both models produce complete elimination of functional LHR in the (–/–) mice. In principle the phenotypes observed in the two models are identical, although the two laboratories producing the mice interpret their findings somewhat differently, in particular as concerns the evidence for or against the functional significance of extragonadal LH/hCG action (*see*, e.g., ref. *14,15*).

The luteinizing hormone knockout (LuRKO) males were born phenotypically normal with testes and other genital structures indistinguishable from their wild-type littermates *(13)*. This indicates, as has been shown with several other models that the fetal population of Leydig cells is able to develop and be functional in the absence of LH action. For instance, the highest increase of rat fetal testicular testosterone level occurs at embryonic days 18 and 19, when there is hardly any LH in circulation *(16)*. Likewise the fetal–neonatal peak in testicular testosterone content is identical in wild-type and gonadotropin-deficient hypogonadal *(hpg)* mutant mice *(17)*, which moreover are normally masculinized at birth *(11)*. The same was observed in *Cα* knockout mice *(7)*, and in mice with targeted disruption of the thyroid-specific enhancer-binding protein (*T/ebp/Nkx2.1)* transcription factor and total absence of development of the pituitary gland *(18)*. Although fetal rat and mouse Leydig cells do express functional *LHR* and their steroidogenesis is responsive to LH stimulation *(19–22)*, their function is not intimately dependent on this stimulus. Several other nongonadotrophic hormones and growth factors have been shown to maintain fetal Leydig cell steroidogenesis *(23–26)*, thus, being able to compensate for missing LH action. In this respect the mouse and rat differ from human, where *LHR* inactivation blocks the intrauterine masculinization process *(2)*. However, human newborn males with inactivating *LH*-β mutation are normally masculinized because placental hCG can compensate for the missing pituitary LH *(3,4)*. Hypogonadism in these individuals begins only at puberty.

Postnatally, testicular growth and descent, as well as external genital and accessory sex organ maturation were blocked in LuRKO males (Fig. 1), as a consequence of the dramatic reduction in the number and size of the adult growth phase of Leydig cells *(12,13)*. Spermatogenesis was arrested at the round spermatid stage, and in this respect the LuRKO model differs from the *hpg* mouse, deficient of both LH and FSH, where spermatogenesis is blocked premeiotically *(11,27)*. The difference is apparently because of normal FSH action in LuRKO mice, indicating that FSH can drive spermatogenesis through meiosis but not

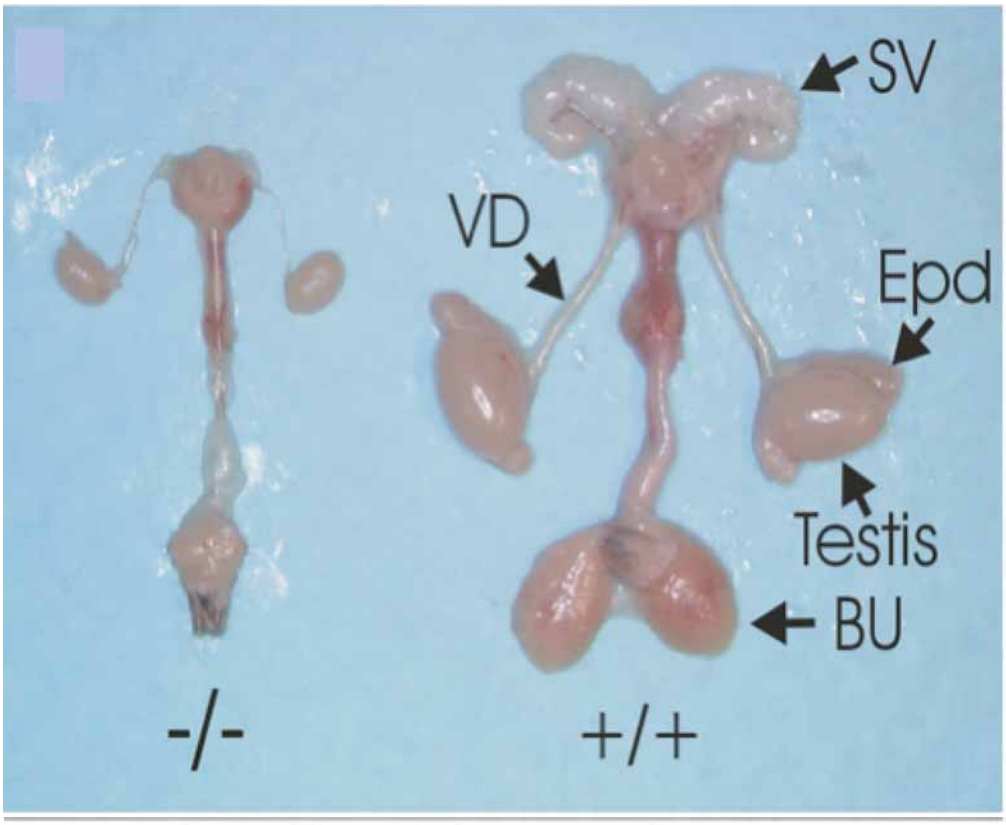


Fig. 1. Testes and accessory sex organs of a (–/–) LuRKO mouse and a (+/+) littermate. VD, vas deferens; SV, seminal vesicle; Epd, epididymis: BU, bulbo-urethral gland. From ref. *13* with permission. (Please *see* color version of this figure in color insert following p. 180.)

Table 1
The Average Testis (wt), ITT, and Serum T in WT, LuRKO, hpg Mice

	Testis wt. (mg)	*ITT (nmol/L)*	*Serum T (nmol/L)*
WT	100–130	500	3–5
LuRKO	20	10	0.6
hpg	6–8	12–20	0.3

The data are extrapolated from the existing literature cited in the text.
ITT, intratesticular testosterone; T, serum testosterone; WT, wild-type; LuRKO, luteinizing hormone knockout mice; *hpg* gonadotropin-deficient hypogonadal mice.

further. In accordance, the LuRKO testes weigh about threefold more than those of the near-totally gonadotropin deficient *hpg* mice (Table 1). The normal masculinization of the knockout mice at birth demonstrated that specific elimination of LH action *in utero* is not detrimental to fetal Leydig cell androgen production, as has also been shown in other experimental models *(17,18,25)*.

To study further the functional defects of the LuRKO males, their testicular expression of selected Leydig-cell specific genes was followed between birth and adulthood *(28)*. Testis weights were similar at birth in control and knockout mice, but remained low in the latter group after 3 wk of life (Table 1). Testicular testosterone content was similar at day 1, but reduced by about 98% by day 70 (Table 1). Whereas the intratesticular testosterone concentration in wild-type mice is 0.5–1 µmol/L, it is only about 10 nmol/L in LuRKO testes. Likewise, whereas the testicular testosterone production in vitro was similar in LuRKO and wild-type testes at birth it was nondetectable in the former mice at 70 d. The expression levels of several Leydig

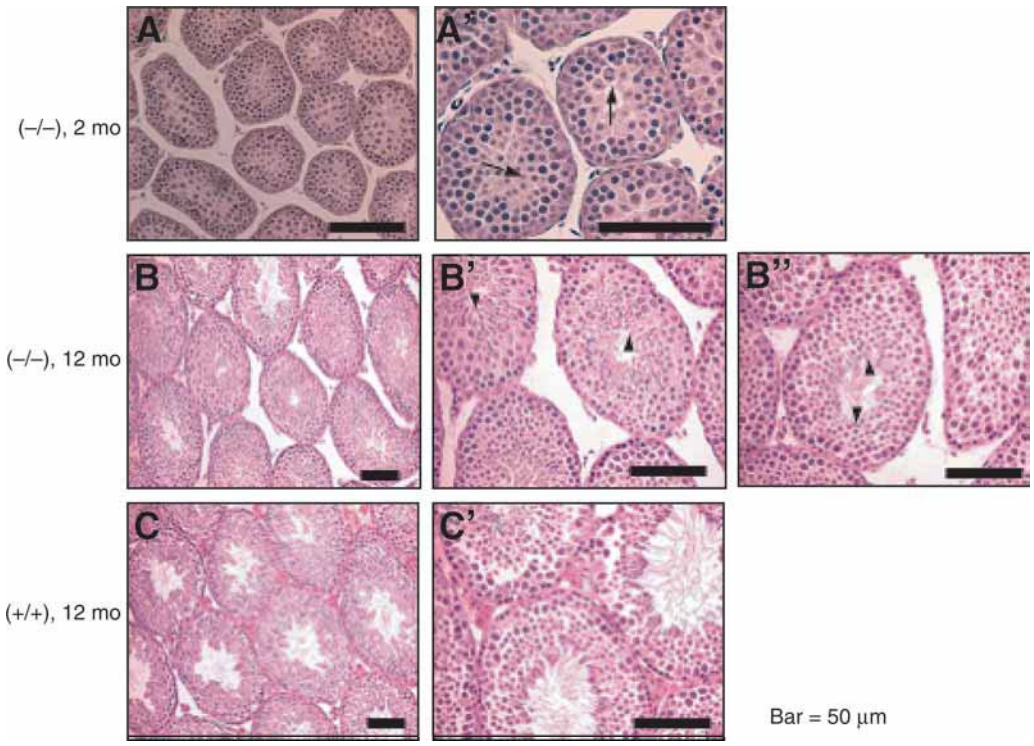


Fig. 2. Representative light micrographs of testis sections from homozygous LuRKO (–/–) and wild-type control (+/+) mice. Samples were taken at the age of 2 mo **(A)** and 12 mo **(B)** and from control wild-type mice at 12 mo **(C)**. The **A', B', B"** and **C'** are views of A, B, and C at high magnification. Arrows and arrow heads indicate round spermatids and elongated spermatids, respectively. Mo, month. From ref. *30* with permission. (Please *see* color version of this figure in color insert following p. 180.)

cell-specific genes, such as *cytochrome P450 side-chain cleavage, 17α-hydroxylase cytochrome P450, 17β-hydroxysteroid dehydrogenase (HSD) type III*, *steroidogenic acute regulatory protein* (*StAR*) and *insulin-like factor 3* were similar in newborn (–/–) and (+/+) testes, but became gradually low or undetectable in the knockout testes during the postnatal development. The only exception was *3β-HSD type I* in peritubular Leydig cell precursors and mesenchymal cells, remaining high in the (–/–) testes. Although, the expression levels of the steroidogenic enzyme genes were low, they were not undetectable, indicating that Leydig cell precursors can maintain a low constitutive steroidogenic activity, and that the effect of LH stimulation on steroidogenesis is not an all-or-none phenomenon. In a similar study on the other *LHR* knockout model, Lei et al. *(12)* observed a suppression of testicular *estrogen receptor-α* expression, no effect on *androgen receptor,* but an increase in that of *estrogen receptor-β*. They also observed an increase in estrogen production of LuRKO testes, which was proposed to arise through Sertoli cell stimulation by the elevated FSH levels in these mice. The expression of the fetal Leydig cell marker *thrombospondin 2* was detectable in the LuRKO testes after puberty *(28)*, indicating that these cells persist until adulthood, as has been suggested previously by morphological evidence *(29)*. However, the relative proportion of these cells of the total testicular mass as well as of all Leydig cells decreases drastically as the testis grows.

When the LuRKO males were studied at the age of 12 mo *(30)*, quite surprisingly, about 60% of the tubular cross-sections demonstrated qualitatively full spermatogenesis up to the elongating spermatid stage (Fig. 2). Because of the low intratesticular testosterone concentration, about 10 nmol/L, which in fact is similar to the circulating concentration in wild-type mice; the finding provides further evidence for the contention that normally, high intratesticular testosterone level may not be absolutely necessary for spermatogenesis. When the action of the residual testosterone was blocked by treatment with the antiandrogen flutamide, the progression from round to elongating spermatids was totally blocked. This step is known to be the most critically androgen-dependent phase of spermatogenesis *(31)*. Hence, even the low testosterone production of the poorly differentiated Leydig cells, deprived of LH stimulation, can in certain situations be sufficient to stimulate the progression of spermatogenesis to

completion. The finding on spermatogenic effect of the low intratesticular testosterone level has implications into the development of male hormonal contraception, based on blockage of gonadotrophin secretion and maintenance of peripheral androgen levels by testosterone substitution therapy. Because the inhibition of gonadotropin secretion by androgen treatment in clinical trials does not result in complete suppression of spermatogenesis *(32,33)*, it is possible that also the residual constitutive androgen production has to be blocked in order to achieve azoospermia in a predictable manner.

Enhancement of LH Action

Enhanced LH action in human, in the form of activating *LHR* mutations, explains the pathogenesis of gonadotropin independent early-onset precocious puberty (testotoxicosis) *(2)*, but curiously no phenotype has been detected in female carriers of these mutations. High gonadotropin action has also been proposed to be a causative factor in some endocrine tumors, for example, ovarian cancer, in women *(34,35)*. Mouse models for enhanced gonadotropin production or action should therefore be useful in search for the elusive female phenotype of LHR overactivation. The phenotypes of male littermates of such mice are informative with respect to response of males to enhanced gonadotropin action, and the Leydig cell phenotypes of mice exposed to elevated LH action will be reviewed next.

LH/hCG Overproducing Transgenic Mice

The first LH overproducing gain-of-function mutation was created by expressing the bovine *LH*-β-*subunit* tethered to the 24-amino acid C-terminal peptide of hCG-β, under the bovine *C*α promoter *(36)*. The transgenic LH-β, when dimerized with endogenous Cα in the pituitary gland, produced moderately elevated levels of LH bioactivity in female, but not in male mice. Hence, although the model has turned out very informative with respect to enhanced LH action in the female (*see* e.g., refs. *37,38)*, no phenotype was found in the male.

Rulli et al. *(39,40)* recently developed transgenic mice which express the *C*α and *hCG*-β subunits under the *ubiquitin C* promoter. The former mouse has no phenotype in either sex, providing further evidence that only dimeric glycoprotein hormones are biologically active. The transgenic *hCG*-β is also expressed in the pituitary gland where it is dimerizes with the endogenous Cα subunit, thus giving rise to approx 40-fold increase in LH/hCG bioactivity in the serum of transgenic females, but only threefold in males. When the two transgenic lines were intercrossed (hCG+ mice), the LH/hCG bioactivity increased over 1000-fold in both sexes. Both the hCG-β+ and double-transgenic hCG+ mice developed clear phenotypes with multiple gonadal and extragonadal tumorigenesis in females *(41)*. Another hCG overproducing mouse line with similar phenotype was recently reported by Matzuk et al. *(42)*.

In male mice, the modest increase in LH/hCG bioactivity induced by the single *hCG*-β expression was not sufficient to bring about a clear phenotype. The mice were fertile and presented only with a mild reproductive phenotype of slightly reduced testis size. In contrast, the double transgenic mice with the highly elevated hCG levels were infertile, apparently because of aggressive behavior and inability to copulate, as a consequence of highly enhanced testicular androgen production *(39)*. Although, the strong LH/hCG stimulation was associated with mild Leydig cell hypertrophy at 60 d of age (Fig. 3), it failed to promote testicular tumors, in stark contrast to the findings in females *(40)*. Despite full spermatogenesis as young adults, progressive degeneration of the seminiferous epithelium was found in many mice when older. The prostate and seminal vesicles were enlarged because of the increased androgen levels, and these structures also caused urethral obstruction and sperm accumulation in distal vas deferens as well as dilated urinary bladder and enlarged kidneys. These pathologies were also consequences of the highly elevated testosterone levels and another reason for the infertility of the mice, not unlike the findings with FSH overexpressing males (*see* "Enhancement of FSH Function"). Despite the early onset of Leydig cell steroidogenic activity, no advancement of puberty was found these mice, which is in contrast to the female hCG+ mice *(40)* and human males with activating *LHR* mutations *(2)*. It can be speculated that the pace of pubertal development and onset of spermatogenesis are already at their maximum in wild-type mice and cannot be advanced by early activation of androgen production.

It was expected that the very high level of hCG would have induced Leydig cell tumors in the hCG+ males, as was the case with the transgenic females and as has been observed as Leydig cell adenomas in men with some activating *LHR* mutations *(43)*. However, the only aberrant finding in Leydig cells of adult hCG+ mice was their mild hypertrophy. In contrast, very prominent Leydig cell adenomas of fetal Leydig cell origin were found in neonatal hCG+ mice (Fig. 3), but these tumors disappeared before puberty *(40)*. Hence, there is a clear difference between fetal and adult Leydig cells in their response to high LH/hCG stimulation. A tumorigenic response in the form of adenomas is only found in the fetal population whereas the adult

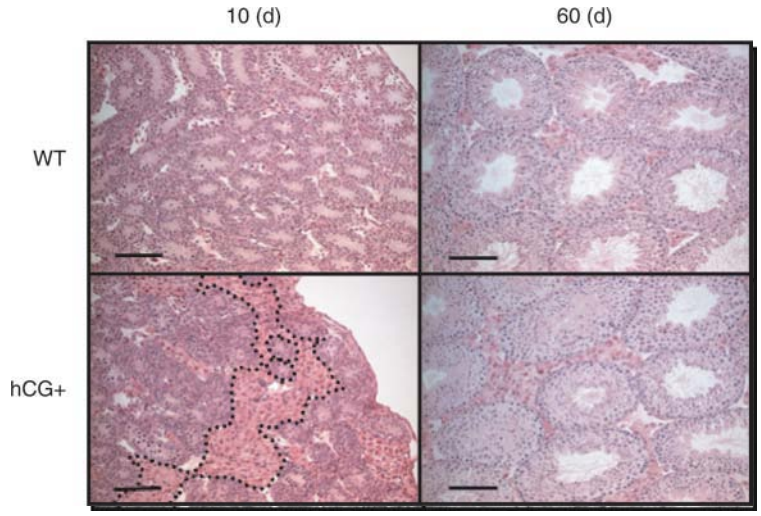


Fig. 3. Testicular histology of 10 and 60 d-old wild-type and transgenic hCG+ mice. The Leydig cell adenoma in the 10-d hCG+ testis is surrounded by a dotted line. Bar = 50 µm. (Please *see* color version of this figure in color insert following p. 180.)

Leydig cells are resistant to any tumorigenic effect of high gonadotropic stimulation *(44)*. In this respect there is a marked sex difference in LH/hCG action. The other surprising finding in hCG+ males was the total resistance of their timing of puberty to the early onset of testosterone production.

GENETIC MODIFICATIONS OF FSH FUNCTION

FSHR is expressed in the testis exclusively in the Sertoli cells. Hence any FSH actions on Leydig cell function must be indirect, through paracrine actions originating from Sertoli cells. Although, such paracrine interactions have been demonstrated in multiple animal experiments, there is no solid evidence in human. The Leydig cell function, as monitored by the onset of puberty and serum testosterone levels, has been largely normal in the three men with inactivating *FSH*-β mutation *(45–47)* and five men with inactivating *FSHR* mutation *(48)*. Only one of the men in the former group presented with delayed puberty and low testosterone levels *(47)*, which may have been because of another causative factor and unrelated to FSH inactivation. One man with activating *FSHR* mutation has been reported *(49)*, but there was no evidence for effect of the mutation on Leydig cell function.

Inactivation of FSH Action

Abolition of FSH action has been achieved in mice by targeted disruption of the *FSH*-β *(50)* and *FSHR (51,52)* genes. Mice with *FSH*-β knockout have reduced testicular size to less than half of normal and their spermatogenesis is quantitatively, though not qualitatively, impaired. Nevertheless, the mice maintain roughly normal fertility. Their Sertoli cell number is reduced *(53)*, in keeping with the known effect of FSH on proliferation of these cells in the immature testis. However, the Leydig cell number, testosterone production and sizes of the androgen-responsive accessory sex glands are normal *(53)*.

FSHR knockout mice appear to have similar phenotype with smaller testis size but maintenance of normal fertility *(51,52)*. More detailed studies have shown that the phenotype of the receptor mutation is more severe, in particular as concerns clear effects on Leydig cell function in adult age *(54–56)*. The two knockout models are indistinguishable from each other and wild-type controls at birth. Whereas no difference in Leydig cell function is seen between the ligand knockout and controls in adult age, the receptor knockout mice have clearly reduced Leydig cell number, testosterone production, levels of several key steroidogenic enzymes, and a converse elevation of serum LH levels. These findings indicate the presence of an FSH dependent paracrine action from Sertoli cells, which stimulates adult Leydig cell development and function. Even a nonliganded FSHR is sufficient, and it may possess sufficient constitutive activity to permit secretion of the paracrine Sertoli cell-derived stimulus of Leydig cells *(56)*. In accordance, the expression of transgenic *FSHR*, but not *FSH*, in the *hpg* background is associated with elevated testosterone production *(10,57)*. Hence, in the presence of LH, FSH is not required for normal pubertal Leydig cell development as long as FSHR are present. The identity of the Sertoli cells derived tropic factor for Leydig cells

remains elusive, but compounds such as desert hedgehog, platelet-derived growth factor, and interleukins have recently been implicated in this regulation *(58–60)*.

Enhancement of FSH Function

Genetically modified mice with enhanced FSH secretion have also been produced. They are intercrosses of two transgenic mice, one expressing the *hCG* α-*subunit* minigene and the other the human *FSH* β-*subunit* gene *(61)*. Depending on the line, mice expressing either low (about 50 U/L) or very high (about 150,000 IU/L) levels of circulating FSH were produced. The low expressor males were fertile and indistinguishable from their control wild-type littermates, indicating that mildly elevated FSH levels do not interfere with testicular function, including that of Leydig cells. Most of the high expressor males were infertile. No differences were found in testicular or epididymal size or morphological appearance. The seminal vesicles were enlarged, consistent with about 20-fold elevation of circulating testosterone level, however, no morphological alterations were found in Leydig cells. Whether the elevated testosterone in the presence of these very high pharmacological levels of FSH indicates activation of an FSH-dependent paracrine loop between Sertoli and Leydig cells or whether it indicates crossreaction of FSH with Leydig cell LH receptors, remains open. The infertility of the males was considered likely to be caused by behavioral defect, in the absence of endocrine hypofunction, and in line with observations on normal Leydig cell function in male patients with pituitary adenomas secreting large amount of bioactive FSH *(62,63)*.

CONCLUSIONS AND FUTURE PERSPECTIVES

Genetically modified mouse models are now available for all main permutations of aberrant gonadotropin action, including knockouts for all gonadotropin subunits and the two receptors, and transgenic mice overproducing LH/hCG and FSH. Many of these mutant mice have been perfect phenocopies of the respective human mutations, for instance the inactivating *FSHR* mutation in both sexes. There are also differences, for instance the precocious puberty of human males with activating *LHR* mutation, but no advancement of puberty in male mice with enhanced hCG production, and a reverse situation in females of both species. As concerns Leydig cell function, the mouse models have elucidated the effects of enhanced LH stimulation, the indirect effects of FSH, and the functional importance of their residual gonadotropin independent androgen production. In most cases, the molecular mechanisms of the species differences in gonadotropin action can be clearly explained.

It is expected that more accurate experimental models for the human mutations in gonadotropin action can be obtained by using knock-in techniques, to achieve identical point mutations with those observed in human patients. As concerns Leydig cell function, it will be intriguing to see whether activating point mutations of *LHR* induce similar phenotypes with the LH/hCG overexpressing transgenic mice. The potential tumorigenic effects of specific activating *LHR* and *FSHR* mutations in an in vivo animal model would also be important in further exploration of the proposed tumor-promoter function of gonadotropins and its relevance to Leydig cell function.

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18 Growth Factors in Leydig Cell Function

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SUMMARY

Leydig cell function is predominantly controlled by gonadotropins. However, a number of intratesticular factors are known to influence Leydig cell steroidogenic function. The presence of growth factors within the testis, their effects on androgen release by the isolated Leydig cells, subnormal reproductive function in animals with disrupted growth hormone/insulin-like growth factor-I secretion, and in acromegalics as well as in Laron syndrome patients clearly indicate that growth factors play an important role in reproduction. It is possible that there might be a concerted effect of intratesticularly produced factors on testosterone secretion. Evidence presented in this chapter indicates that pituitary luteinizing hormone is absolutely essential for Leydig cell endocrine function, but growth factors are required for the full effect of luteinizing hormone on androgen secretion. Also, there are indications that a number of growth factors modify gonadotropin synthesis and release from the pituitary gland. Thus, growth factors can exert endocrine as well as paracrine/autocrine effects in controlling the pituitary and testicular functions in mammals.

Key Words: Acromegaly; growth hormone; insulin-like growth factor-I; Laron syndrome, pituitary–testicular function; testosterone.

INTRODUCTION

The mammalian testis is a complex organ with two major functions, one to produce viable spermatozoa for fertilization of the egg and the other to produce steroids as well as paracrine/autocrine factors to assist spermatogenesis, maintain accessory reproductive structures, and sexual behavior. Leydig cells and Sertoli cells within the testis are the major endocrine components, which have the capacity to synthesize and secrete steroids and peptides. In this chapter, some data related to production of growth factors by different cell types in the testis and the control of Leydig cell endocrine function by some of the known intratesticular growth factors are presented.

It is firmly established that Leydig cell function is predominantly controlled by the action of LH secreted by the pituitary gland. However, there are studies suggesting that follicular stimulating hormone (FSH) increases Leydig cells function *(1–3)*. It has been shown that treatment of immature hypophysectomized rats with either purified FSH or recombinant human FSH induces Leydig cell hyperplasia, hypertrophy, and increases the number of LH receptors as well as LH receptor messenger RNA (mRNA) levels *(4–6)*. Moreover in men, administration of recombinant human FSH increased circulating and spermatic venous blood testosterone levels *(7)*. Furthermore, it was shown in a man that a mutation of the *FSH*-β gene resulted in attenuated testosterone secretion *(8)*. In male FSH receptor gene knockout mice, there was a delay in sexual maturity that was accompanied by reduction in fertility as evidenced by mating studies *(9)*. These results suggest that FSH has a role in the control of Leydig cell function. However, some studies have questioned the importance of FSH in male reproduction. In *FSH*-β gene *(10)* and FSH receptor gene knockout mice *(11)*, the Leydig cell function is normal and the animals are fertile.

Another pituitary hormone that influences steroidogenesis of Leydig cells is prolactin (PRL). Naturally occurring increases in circulating levels of testosterone in men during sleep are preceded by elevations in

*Deceased.

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plasma concentrations of PRL *(12)*. Treatment of hypophysectomized rats with PRL potentiates the effect of LH on testosterone production as well as increases in the activities of 3β-(HSD) hydroxysteroid dehydrogenase in rats *(13)* and 17β-HSD in mice *(14)*. Administration of PRL to adult male rats increases plasma testosterone levels *(15)*. These studies indicate that PRL influences testicular function. Furthermore, the ability of LH to induce testosterone secretion is attenuated in rats actively immunized against PRL *(16)*; the presence of specific binding sites for PRL on Leydig cells *(17)* and the maintenance of testicular LH receptors by PRL *(18)* strongly suggest that PRL might be required for testosterone biosynthesis. Numerous intratesticular factors including a number of growth factors influence the secretion of androgens by the Leydig cells. In this review, the influence of some of the known growth factors on Leydig cell endocrine function is briefly described.

Growth Factors

It is demonstrated that many growth factors including transforming growth factor (TGF)-β, TGF-α, epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and insulin-like growth factor (IGF) as well as IGF-binding proteins (IGFBPs) affect the steroidogenic function of the testis. The authors briefly examine the specific functions of these growth factors.

Transforming Growth Factor-β

TGF-β has been demonstrated to be present in three forms (β1–β3) in the testis of various mammals. Three membrane-bound receptors, types I–III (also called betaglycan) are involved in binding of TGF-β *(19–21)*. It is believed that type I receptor requires type II receptor for signaling *(22–24)* and type III receptor has no major role in TGF-β signaling, but it aids in binding of TGF-β2 to type II receptor and increases the cell response to this peptide *(24)*.

Sertoli cells and peritubular cells isolated from immature rats contained TGF-β mRNA *(25)*. It has been shown that Sertoli cells secrete TGF-β1, and peritubular cells produce both TGF-β1 and TGF-β2 *(25,26)*. TGF-β3 mRNA was present in substantial amounts in testes of mice *(26)*. In the adult mouse testis, germ cells express TGF-β1 mRNA *(27)*. In immature pig testis, both Sertoli cells and Leydig cells express TGF-β1 mRNA *(28)*. Rat fetal Leydig cells also express TGF-β1 mRNA *(29)* which seems to disappear in Leydig cells of adult animals *(30)*.

A number of in vitro studies have shown that TGF-β inhibits testicular steroidogenesis. Addition of TGF-β to isolated porcine Leydig cells resulted in a significant decrease in LH/human chorionic gonadotrophin (hCG) receptor numbers without affecting the Leydig cell proliferation *(31)*. Similar results were obtained in studies in rats with a reduction in cAMP and testosterone responses to hCG treatment *(32,33)*. It has been suggested that TGF-β has a biphasic effect on hCG action associated with testosterone secretion. In porcine Leydig cells, it has a stimulatory effect at low dose and an inhibitory effect at high concentration *(34)*. TGF-β decreases cytochrome P-450scc and 17α-hydroxylase *(35)*, resulting in reduced Leydig cell capacity to secrete testosterone. However, normal development of the testis is observed in *TGFβ1* gene-disrupted mice *(36,37)*, suggesting that this peptide might have little influence on testicular development. Because the majority of TGF-knockout mice do not survive postnatally *(38–40)*, because of cardiomyopathy and neural tube defects, no information is available related to the adult testicular endocrine function in these mice. In transgenic mice overexpressing TGF-β1, the seminiferous tubular basement membrane was thickened and the Leydig cells were prominent *(41)*, but their steroidogenic function remains unknown.

TGF-α and EGF

EGF family of growth factors consists of EGF, TGF-α, and amphiregulin. These peptides bind to a single EGF receptor (EGFR) with tyrosine kinase activity *(42)*. EGF binding-sites are present in the human *(43)*, rat *(44)*, mouse *(45)*, and porcine Leydig cells *(46)*. Furthermore, it has been shown that EGFRs are present in Sertoli cells of immature and mature rats *(44)*. During early puberty the majority of EGFR mRNA was identified in peritubular cells *(47)*.

Several studies have shown that EGF treatment affects testicular steroidogenesis. In MA-10 mouse Leydig cell tumor line, EGF stimulated progesterone production had potentiated the steroidogenic effect of hCG *(48)*. Treatment of MA-10 Leydig cells with EGF significantly increased the levels of progesterone, StAR protein, and StAR mRNA in a time- and dose-dependent manner *(49)*. EGF alone has been shown to moderately increase the testosterone secretion in isolated human *(50)*, rat, and mouse Leydig cells *(51)*. These investigators have also shown that EGF directly stimulates the output of C19-steroids (testosterone and androstenedione) and C21-steroids (progesterone, 17α-hydroxyprogesterone, and 20α-hydroxypregn-4-en-3-one). EGF has been shown to increase the gonadotropin effect on androgen

formation through an increase in the availability of cholesterol substrate in the mitochondria and an increase in the activity of 3β-HSD in isolated immature porcine Leydig cells *(46)*. In constrast to the earlier findings, EGF has been shown to attenuate the ability of hCG to stimulate steroidogenesis in MA-10 Leydig cells *(52)* and in cultured Leydig cells obtained from adult rats *(53)*.

Because TGF-α knockout mice had no alterations in testis phenotype, and EGFR knockout mice had a transient decrease in the relative amount of interstitial cells before birth and no significant inhibition of testis growth *(54)*, it is not clear whether Leydig cell functions are affected in these mice.

Fibroblast Growth Factor

At least seven polypeptides belong to the FGF family. They are acidic FGF (aFGF or FGF-1), basic FGF (bFGF or FGF-2), int-2 (FGF-3), Kaposi sarcoma FGF (K-FGF or FGF-4), and FGF-5, FGF-6, and keratinocyte growth factor (KGF or FGF-7) *(55)*. Additionally, a number of other FGFs (FGF-8–24) were identified *(56–58)*. These factors are present in many tissues and have many functions, importantly in stimulation of cell division and differentiation of cells of embryonic mesoderm and neuroectoderm, neuronal growth, and angiogenesis *(55)*. The FGF receptor (FGFR) is a single-chain transmembrane glycosylated protein with a cytoplasmic tyrosine kinase and two or three extracellular immunoglobulin-like domains *(59,60)*. There are four recognized FGFRs. They are FGFR-1/flg, FGFR-2/bek, FGFR-3, and FGFR-4 *(60,61)*. The receptors for FGF are present in isolated testicular cells of neonatal rats *(62)*, and in immature rat *(62,63)* and porcine Leydig cells *(64)*.

The bFGF mRNA is identified in mouse Leydig cells, and germ cells *(65,66)*. Also, this growth factor is present in human *(67)* and bovine *(68)* testis and a FGF-like factor is secreted by isolated rat Sertoli cells *(69)*. bFGF mRNA levels have been shown to decrease at sexual maturation *(47)*. Freshly isolated peritubular, Leydig and Sertoli cells from rats express bFGF mRNA *(69)*. Similarly, cultured porcine Sertoli and Leydig cells contains bFGF mRNA *(70)*.

It has been demonstrated that FGF inhibited the LH-stimulated androgen production by isolated testicular cells obtained from neonatal rats *(71)*. In cultured immature rat Leydig cells, bFGF reduces LH/hCG receptor number and gonadotropin-stimulated testosterone secretion *(72)*. These investigators also showed that this growth factor reduced the activities of 3β-HSD *(73)*, 17β-HSD *(64)*, and 5α-reductase enzymes *(74)*. With respect to the LH receptor numbers and 3β-HSD activity, it is believed that the effects of bFGF on immature rat Leydig cells are biphasic, inhibitory at low dose, and stimulatory at high dose *(75)*. However, aFGF in high concentrations inhibited the Leydig cell function *(72)*.

In cultured porcine Leydig cells, bFGF increases basal testosterone release *(64,76)*. However, it has been shown that long-term treatment with bFGF reduces the gonadotropin receptor number and no change in hCG-induced testosterone production *(77)*. It was also reported that bFGF increases gonadotropin-stimulated androgen secretion associated with increases in the activities of 3β-HSD and 17β-HSD *(76,78)*.

As a result of these controversies, apparent species differences and inconsistent data from in vivo studies related to the influence of FGF on Leydig cell steroidogenic function, the physiological role of these factors is not clear. Recently, FGF-deficient and FGF-overexpressing transgenic mice were developed *(79,80)*. The information related to the testicular endocrine function in these mice will certainly add information related to the understanding of the role of FGF in male reproduction. It has also been demonstrated that FGFs are critically involved in the development of gonadotropin-releasing hormone (GnRH) neurons *(81)*. In transgenic mice expressing a dominant-negative FGFR mutant gene targeted to GnRH neurons, the number of these neurons was reduced by 30% *(82)*. These mice exhibited delayed puberty, reduced litter size, and early reproductive senescence. Therefore, FGFs seems to be one of the important components involved in the formation and maintenance of the GnRH neuronal system, which is essential for normal pituitary as well as reproductive functions in mammals.

Platelet-Derived Growth Factor

PDGF is widely distributed in the body and is made up of two polypeptide chains. There are PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC, and PDGF-DD isoforms *(83)*. It has been shown that PDGF acts through two receptor subunits, α and β. Both α- and β- receptor subtypes are expressed in Leydig cells of adult rats *(84)*. In cultured immature rat Leydig cells, PDGF inhibits the basal 3β-HSD activity and hCG-stimulated testosterone formation *(85)*. In contrast, PDGF-BB potentiated the LH effect on testosterone production in cultured adult rat Leydig cells *(86,87)*. In PDGF-deficient mice, at 42 d of age, there was a complete loss of Leydig cells with absence of circulating testosterone *(88)*, suggesting that PDGF might have a physiological role in Leydig cell morphogenesis and function. The

Table 1
Effects of Growth Factors on Leydig Cell Endocrine Function

Growth factors	*Effect on Leydig cells*	*References*
TGF-β	The testosterone (T) response to gonadotropin treatment decreased. In isolated porcine Leydig cells, TGF-β has a biphasic effect on hCG action. TGF-β at low dose, T increased; at high concentration, T decreased.	*32–35*
TGF-α and EGF	Testicular steroidogenesis increased.	*48–53*
FGF	Generally it inhibits T secretion in cultured neonatal and immature rat Leydig cells. In isolated immature rat Leydig cells, bFGF has biphasic action, at low dose, T decreased and at high dose, T increased. aFGF at high concentrations, androgen secretion decreased. In cultured porcine Leydig cells, bFGF increases basal T release.	*71–76*
PDGF	PDGF inhibits the hCG-stimulated T release in immature Leydig cell cultures. PDGF-BB increased the LH effect on T production in cultured adult rat Leydig cells.	*85–86*

main effects of various growth factors on Leydig cell function are summarized in Table 1.

Insulin-Like Growth Factor-I

IGF-I and IGF-II are single-chain polypeptides with a significant role in growth and differentiation. IGF-I affects postnatal growth and IGF-II influences fetal growth. The actions of these peptides are through type I (IGF-IR), type II (IGF-IIR), and insulin receptors. IGF-IR is the main mediator of IGF-I action. The biological effects of IGFs are profoundly influenced by six IGFBPs found in the circulation and in organs. Despite the fact that liver is the major site of synthesis of IGF-I, a number of studies have shown that the testis produces IGF-I. It has been shown that rat testis contained immunoreactive IGF-I and IGF-I mRNA *(89,90)*. IGF-I is localized in Sertoli cells of the rat testis *(89,91)*. Furthermore, IGF-I was identified in human Sertoli and Leydig cells as well as in primary spermatocytes *(92)*. The IGF-I receptors were identified in Sertoli cells *(93)*, Leydig cells *(94)*, secondary spermatocytes, and spermatids *(92)*. Additionally, testis contain IGFBPs. It has been demonstrated that IGFBPs are produced by Sertoli, Leydig, and peritubular cells. IGFBP-2, -3, and -4 mRNAs are found in testes of rats *(95–97)*. The peritubular cells of the testis produce IGFBP-2, whereas peripubertal and Sertoli cells synthesize IGFBP-3 *(95,96)*. Rat Leydig cells express IGFBP-2, -3, and -4 genes *(97)*. Additionally, seminiferous tubules contain low amount of IGFBP-3. It has been shown that IGFBP-1 is not found in the rat testis *(97)*. Similarly, human testis does not express *IGFBP-1* gene, but *IGFBP-2* mRNA was detected in Sertoli and Leydig cells *(98)*. *IGFBP-3* was present only in endothelium of testicular blood vessels, whereas *IGFBP-4* and *IGFBP-5* were present in Leydig cells, and *IGFBP-6* gene was expressed in some peritubular cells of the human testis *(98)*. It is believed that *IGFBP-3* and *IGFBP-4* inhibit IGF-I-induced testosterone production by purified Leydig cells *(97)*. In male mice with disruption of *IGFBP-2* or *IGFBP-6* genes, reproduction is not substantially affected *(99,100)*. However, in contrast, it was shown that fertility is affected in transgenic mice overexpressing the *IGFBP-6* gene *(101)*. Furthermore, in male transgenic mice overexpressing the *IGFBP-1* gene, there were reductions in spermatogenesis and testosterone secretion *(102)*. These studies suggest that IGFBP-1, and possibly IGFBP-6 might play a role in spermatogenesis as well as Leydig cell endocrine function.

The most extensively studied growth factor that has a significant impact on reproduction is IGF-I. IGF-I is produced mainly in the liver in response to growth hormone (GH) action and reaches its targets through circulatory system. Also, it is locally produced in different organs and the secretion is regulated independently of GH. The production of testicular IGF-I is believed to be under the control of gonadotropins. The secretion of testicular IGF-I increases following FSH and LH treatments *(103,104)*. It is documented that locally synthesized IGF-I plays an important role in the intricate paracrine control of different types of somatic cell

functions in the gonads. Studies in mice with isolated GH deficiency or targeted disruption (knockout) of genes coding for GH receptor (GHR), IGF-I, or IGF-I receptors indicate that IGF-I signaling is definitely required for sexual development and sexual maturation *(105–108)*. However, GH deficiency does not abrogate fertility in the mature individual *(105–108)*. In most of the mammals adequate action of GH/IGF-I is essential for the normal process of maturation and for normal reproduction *(109–114)*.

Systemic and locally produced IGF-I can influence the hypothalamic–pituitary–gonadal axis. However, data obtained in transgenic animals overexpressing GH and in human acromegalics with increased GH secretion indicate that chronic elevation of GH and IGF-I levels above the normal range can interfere with reproduction. Thus, studies in children with congenital GH deficiency or GH resistance *(115,116)* and in adults with GH secreting pituitary tumors *(117,118)* indicate a relationship of the somatotropic axis and reproduction. Furthermore, GH or IGF-I treatment can influence the gonadotropin release *(119–123)*, gametogenic and steroidogenic functions of the gonads *(124–128)*, sperm motility *(129,130)*, erectile function *(131,132)*, and fertility *(133–136)*. It has been shown that treatment with recombinant human GH can augment the effects of exogenous gonadotropins in male patients treated for infertility *(137)*. It has also been shown that GH promotes sexual development in children with GH deficiency *(138,139)*. Therefore, physiological amount of GH/IGF-I secretion are required for normal reproduction.

GH/IGF-I acts at multiple sites including action at the level of gonadotropin receptors of testicular Leydig cells *(128,140)*, and on penile growth *(141)*. It has been demonstrated that gonadal steroids promote GH release *(142,143)*, and GH acts synergistically with sex steroids to promote somatic growth and physical maturation that precede and accompany pubertal development *(138,139)*. In the immature male rat, GH acts directly through GHRs on Leydig cell progenitors to stimulate activity of enzymes involved in androgen production *(142)*. It should be recognized that there are some controversies regarding the role of GH/IGF-I in testicular function. It has been shown that treatment of GH to GH-deficient young adult male patients resulted in significant increases in total and free IGF-I levels, but had no significant effect on the basal and hCG-stimulated levels of androgens *(144)*. Therefore, this study suggested that the effects of GH treatment do not appear to involve major alterations in testicular steroidogenic function. Furthermore, it was also shown in baboons that treatment of GH or IGF-I did not alter gonadotropin stimulation of testicular function *(145)*. In cynomolgus monkeys, long-term treatment of GH did not alter spermatogenesis *(146)*, and suppression of GH by active immunization against GHRH failed to affect ongoing spermatogenesis in rats *(147)*. Therefore, it is important to emphasize that not all effects of GH are stimulatory and the duration of exposure of the hypothalamo–hypophyseal–gonadal system to GH/IGF-I might influence the secretions of gonadotropins and androgens.

GH/IGF-I Deficiency

Ames dwarf mice are deficient in GH/IGF-I secretion *(148)*. It has been shown that administration of bGH to these mice induces IGF-I secretion *(148)*. In these mice, GH treatment enhanced plasma LH levels, and the effect of GnRH on LH secretion was significantly increased, but this LH response was lower than in normal siblings who previously received vehicle. Pretreatment of dwarf mice with GH resulted in increased production of androstenedione and testosterone by the isolated testis treated with hCG. These results indicated that the alterations in hypothalamic–pituitary–testicular function in GH-deficient mice are because of the lack of IGF-I secretion. However, Ames dwarf mice are also PRL and thyroid-stimulating hormone (TSH)-deficient and therefore, some of the effects observed in Ames dwarf mice might also have been as a result of absence of PRL and thyroid hormones. Snell dwarf mice similar to Ames dwarfs are GH-, PRL-, and TSH-deficient and their testicular weights, seminiferous tubular diameter, and germ cell numbers are reduced *(149)*. However, administration of GH (resulting in IGF-I secretion) during the postnatal development resulted in normalization of these parameters *(149)*. Additionally, treatment with GH, stimulated the fertility rate in Snell dwarf mice *(150)* suggesting that GH/IGF-I play a significant role in male reproduction. As a consequence of mutations in the Prop-1 locus in Ames dwarf mice and in the Pit-1 locus in Snell dwarf mice, the somatotrophs, lactotrophs, and thyrotrophs fail to develop.

In GH-receptor gene-disrupted (GHR-KO) mice, plasma IGF-I is undetectable *(113,114)*; hence these mice are a good experimental animal model of human Laron syndrome (GH insensitivity). The hypothalamic–pituitary–gonadal function of GHR-KO mice was evaluated in considerable detail. The authors' investigation related to male puberty has shown that the balano–preputial separation was delayed by 5 d in these mice *(112)*. It is known that preputial separation is an external sign of pubertal development in male

rodents *(151)*. Additionally, the normal, significant increase in the weights of seminal vesicles during maturation was postponed in GHR-KO mice *(112)*. The elongated spermatids appeared later in testes of GHR-KO mice relative to testes of normal mice. In addition, weights of testes, and epididymis were significantly reduced in GHR-KO mice. The intratesticular testosterone levels and the testosterone response to LH treatment was decreased in these mice *(112)*. These results suggest that the absence of IGF-I in circulation delays the normal course of sexual maturation in male GHR-KO mice and that IGF-I plays a role in the initiation of puberty in male mice. Similarly, it is also known that sexual maturation is delayed in men with Laron syndrome *(115,116)*. This clinical syndrome is a result of mutated GHR genes with subsequent resistance to GH and IGF-I-deficiency. GHIGF-I are believed to be involved in the regulation of pubertal growth spurt and sexual maturation. It has been proposed that peripherially produced IGF-I might act within the central nervous system to trigger sexual maturation *(123)*.

In adult male GHR-KO mice, the size of testes is reduced approximately in proportion to the difference in body weights between normal and GHR-KO mice *(113,114)*. Morphometric studies have shown that the length and diameter of seminiferous tubules and the percent of volume density of Leydig cells are reduced in young adult GHR-KO mice *(114)*. Although, the basal plasma LH levels were similar in GHR-KO and in normal mice, the plasma LH response to GnRH treatment was significantly reduced *(113)*. It is interesting to note that the plasma FSH levels were significantly reduced whereas plasma PRL levels were elevated in GHR-KO mice *(113,114)*. It has also been shown that plasma testosterone response to LH treatment was attenuated in GHR-KO mice, whereas circulating androstenedione levels were not different than in their normal siblings *(114)*. This suggests that within the testes of GHR-KO mice, 17β-HSD, the key enzyme that converts androstenedione to testosterone is defective or less responsive to exogenous LH. In contrast to GHR-KO mice, in *IGF-I* gene-disrupted mice, the male sex accessory structures were diminutive and the animals infertile *(107)*. However, in GHR-KO mice fertility is reduced, but not totally suppressed *(113)*. Additionally in IGF-I-null mice, the in vitro testosterone response to LH treatment was attenuated *(107)*, and the expressions of some of the steroidogenic enzymes mRNA levels associated with testosterone biosynthesis were reduced *(152)*. The mechanism responsible for the maintenance of fertility in GHR-KO mice is unknown but it is likely to be related to GH-independent production of IGF-I within the testis, which might aid reproduction in these mice. In GH-deficient dwarf rats, GH treatment elevated IGF-I secretion and increased the total number of viable spermatozoa *(153)*. These findings and alterations in the neuroendocrine and testicular function in GHR-KO mice indicate a role of systemic IGF-I in male reproduction.

The consequences of reduction or absence of IGF-I secretion in various animal species and in humans *(106,107,112–114,124,129,141,148,149,152,154–162)* is summarized in Table 2.

Maintenance of fertility in GHR-KO mice might have been because of an effect of PRL. It is possible that, as a compensatory mechanism for GH resistance, the elevated PRL secretion in GHR-KO mice *(113)* might have maintained normal secretion of LH and Leydig cell function, which maintained fertility in these mice. Similarly, in *GHRH-R* gene mutated *lit/lit* mice with isolated GH deficiency, spermatogenesis and testosterone secretion are normal *(159,160)*. However, these mice are subfertile because of a defect in their sexual behavior *(159,160)*. Additionally, it has been shown that male PRL receptor-deficient mice had no major defect in fertility *(163)*. Thus, isolated deficiency of either GH or PRL secretion seems not to affect fertility in the male, whereas combined deficiency of GH/IGF-I and PRL affects reproduction.

The IGFBPs have profound effects on IGF-I action. Therefore, the ratio of IGFBPs and IGF-I within the body might play an important role in the action of IGF-I on the neuroendocrine–gonadal system. The effects of exogenous IGF-I on gonadal function have been evaluated in GHR-KO mice that have a systemic IGF-I deficiency. Acute treatment of GHR-KO mice with IGF-I unexpectedly suppressed the LH-induced testosterone secretion in *GHR* gene-disrupted mice *(164)* (Chandrashekar et al. unpublished data). The total testosterone response of the isolated testes of normal siblings to LH treatment was higher than the response of the testes of GHR-KO mice. Incubation of testes of normal mice with IGF-I enhanced the total testosterone release, whereas the testes of GHR-KO mice were unresponsive to IGF-I treatment. IGF-I did not enhance the LH effect on the total testosterone release. The inhibitory in vivo effect of IGF-I on LH action on testosterone secretion in GHR-KO mice and the increased total testosterone response of isolated testes of normal mice to IGF-I treatment suggest an interference of exogenous IGF-I action in vivo. The differences between the in vivo and in vitro effects of exogenous IGF-I suggest that a proper ratio of IGF-I

Table 2
Effects of Reduction or Absence of Peripheral IGF-I Secretion on Male Reproduction in Some Animals and in Humans

Type	*Neuroendocrine and testicular functions*	*References*
GH-deficient, *dw/dw* rats	IGF-I secretion decreased. Normal pituitary and circulating FSH and LH levels. Pituitary PRL content increased. Normal spermatogenesis and androgen secretion. These rats are subfertile.	*124,129,154*
GH-deficient, *rdw/rdw* hereditary rat	Plasma FSH and LH levels were unchanged; pituitary and serum PRL concentrations decreased. Hypothyroid and infertile.	*155–157*
GH-deficient Snell, *dw/dw*, and Ames, *df/df* mice	Decrease or absence of circulating IGF-I secretion. Plasma LH and FSH levels decreased. Also, these mice are PRL- and TSH-deficient. The number of Leydig cells, testosterone (T) secretion and sperm production reduced. These mice are subfertile or fertile depending on genetic background.	*148,149,158*
GHRH-R-deficient, *lit/lit* mice	GH-deficient. Spermatogenesis and T secretion are normal. However, these mice are subfertile because of defect in their sexual behavior.	*159,160*
IGF-I gene null, –/– mutants	IGF-I-deficient. Reduced spermatogenesis. Total Leydig cell numbers decreased and they fail to mature resulting in reduced circulating T levels. Absence of mating behavior. Males are infertile.	*107,152*
GHR gene knockout –/– mice	Low or undetectable plasma IGF-I levels. Puberty is delayed in males. LH response to GnRH treatment reduced. FSH secretion decreased. Testicular LH and PRL receptor numbers reduced. PRL secretion increased. Fertility is reduced in male mice.	*106,112–114*
Laron syndrome in humans; *GHR* mutation	Primary IGF-1 deficiency and GH resistant. Puberty is delayed in male patients.	*141,161,162*

and IGFBPs might be required for the normal testosterone secretion in GHR-KO mice. It has been shown that the circulating IGFBP-1, 2, and 3 are reduced in GHR-KO mice *(165)*. It is known that IGF-I increases the synthesis and secretion of IGFBPs and transgenic mice expressing *IGFBP-1* gene are infertile *(102)*, and that *IGFBP-3* inhibits Leydig cell steroidogenesis *(97)*. These observations suggest that IGFBPs might have a vital role in the action of IGF-I and male reproduction.

Excess GH/IGF-I

Transgenic male mice expressing the metallothionein-I (MT)–hGH gene construct are fertile. However, their seminal vesicles are significantly enlarged *(166)*. The enlargement of the seminal vesicles might have been because of the PRL-like activity of the secreted hGH *(167)*. In young adult transgenic mice, the plasma testosterone levels and sperm production per gram of testis are similar to those observed in normal mice *(166,168)*. In these transgenic mice, plasma LH levels were significantly increased *(168–170)*. The expression of LHβ mRNA in the pituitary gland was enhanced *(171)*. Despite this increase in LH secretion, the normal basal plasma testosterone levels suggest that the Leydig cells of these mice are unable to respond to increased LH secretion. This might have been because of the PRL-activity of hGH and hyperprolactinemia has been shown to significantly affect testicular steroidogenesis *(172)*. Additionally, the pituitary–hypothalamic– testicular function was unaltered in the male transgenic mice expressing the bovine *GH* gene with the mouse MT promoter *(173)*, suggesting the importance of the PRL-like effect of hGH in mice expressing the *hGH* gene.

Transgenic mice expressing the *hGHRH* gene *(174)*, secrete homologous mouse GH, and these mice are a good animal model for human acromegaly. These animals produce large amounts of GH and the pituitary is enlarged *(175)*. The source of GH is from the *in situ* pituitary gland, and these animals secrete high levels of IGF-I *(176)*. In these transgenic mice, the testicular weights were significantly increased. Although, the basal LH levels were similar in transgenic mice bearing the *hGHRH* gene and in their normal siblings, the LH response to GnRH treatment was

Table 3
The Consequences of Excess GH/IGF-I Secretion in Male Transgenic Mice Expressing Various GH Genes and in Acromegalic Patients

Type	*Pituitary and testicular functions*	*References*
Mice expressing *MT-hGH* gene	Plasma LH and pituitary LHβ mRNA increased. Attenuated plasma LH response to GnRH treatment. Normal testosterone (T) secretion. These mice are hypoprolactinemic. Fertility reduced because of decreased sperm production.	*166,168–171*
PEPCK-hGH transgenic mice	Fertile	*166*
MT-hGH.V (placental variant) hybrid gene expressing mice	Fertility reduced as a result of decreased sperm production.	*166*
MT-bGH and *PEPCK-bGH* transgenic mice	Normal pituitary and testicular functions. Fertile.	*166,173*
Mice expressing *MT-hGHRH* gene	Plasma LH response to GnRH treatment increased. Larger testis and T secretion increased.	*166*
Acromegalic patients	Subnormal testicular function. GH or IGF-I treatment improved T secretion. Many patients are hyperprolactinemic.	*177,178,180*

MT, mouse metallothionein-I promoter; PEPCK, phosphenolpyruvate carboxykinase promoter.

attenuated in transgenic mice. Excess secretion of GH/IGF-I resulted in significant increases in testosterone levels. Additionally, the expression of *hGHRH* gene increased both androstenedione and testosterone secretions. The increased secretions of androgens might have been because of the increased sensitivity of the Leydig cells to LH action. Thus, excess of homologous, endogenously secreted GH and IGF-I positively influences testicular steroidogenesis *(176)*, clearly indicating the influence of IGF-I in testicular endocrine function.

Gonadal function is commonly impaired in human acromegaly. The pituitary of these patients secretes large amount of GH, and the levels of GH and IGF-I are elevated. In normoprolactinemic, acromegalic males, although the gonadotropin response to GnRH treatment was normal, the testosterone secretion was found to be low *(177)*. Furthermore, experimental short-term suppression of GH and IGF-I secretion improved the testicular function in men with acromegaly *(178)*. Additionally, suppression of GH secretion by a somatostatin analog resulted in a blunted LH response to GnRH treatment *(178,179)*. These studies suggest that excess secretion of IGF-I affects the neuroendocrine and testicular functions in animals and human. The effects of excess GH secretion in various transgenic mice and in human *(166,168–171,173, 177,178,180)* are summarized in Table 3.

Ghrelin

Ghrelin is an endogenous ligand that binds to the GH secretagog receptor and it has been shown that it mainly acts centrally to regulate GH secretion by the pituitary gland and food intake *(181–183)*. As ghrelin can influence the growth and energy balance *(184–187)*, it is suspected that this peptide can function as a growth factor. Therefore, the authors present some information related to its effects on the testicular endocrine function.

A testis-specific *ghrelin* gene is expressed in the mouse *(188)*, rat *(189)*, and in human testis *(190)*. In the rat testis, ghrelin is expressed in Leydig cells at advanced stages of maturation *(189,191)*. Similarly, the *ghrelin* gene is strongly expressed in mature Leydig cells of the human testis *(192)*. It has been shown that ghrelin expression in the testis is under the control of pituitary LH *(183)*.

It has been demonstrated that ghrelin significantly inhibits hCG- and cAMP-stimulated testosterone secretion *(189)*. In vitro studies have revealed that this inhibitory effect of ghrelin on testosterone secretion was associated with a significant decrease in hCG-stimulated levels of the mRNAs encoding some key steroidogenic enzymes, namely the P450 side-chain cleavage, 3β-HSD, and testis-specific 17β-HSD type III *(189)*. Also, ghrelin decreases testicular steroid acute regulatory (StAR) protein levels *(189)*. However, the ability of ghrelin to inhibit hCG-stimulated testosterone secretion in vitro is in contrast with the stimulatory influence of pituitary LH on testicular ghrelin expression *(191,193)*. It has been suggested that ghrelin might participate in the autolimitation of testicular testosterone responses to LH/hCG action *(191)*. Thus, ghrelin might influence Leydig cell endocrine function.

However, it was shown that ghrelin-null mice displayed no reproductive abnormality *(194)*, questioning the physiological role of ghrelin involvement in mammalian reproduction.

CONCLUSIONS

Although the pituitary gonadotropins play a dominant role in the maintenance of Leydig cell function, the authors have reviewed the evidence that growth factors exert an important, primarily paracrine influence on male reproduction. The presence of these factors in various testicular cell types, subnormal reproductive function in animals with disrupted IGF-I secretion, and in acromegalic as well as Laron syndrome patients clearly indicate that these growth factors might play important roles in reproduction. Furthermore, impairment of fertility in male transgenic mice overexpressing IGFBP-1 or IGFBP-6 suggests that these and possibly other binding proteins might be involved in the intricate causes of infertility.

Much of the information related to the effects of these factors was obtained from in vitro experiments. Within the testis, communication between different cell types through their secretions would certainly affect the efficacy of the growth factors and the LH action on testicular endocrine function. Therefore it is important to confirm the in vitro data with in vivo studies in order to be certain about the precise effects of growth factors before contemplating their clinical use. It is possible that there might be a concerted effect of intratesticularly produced factors on androgen secretion. In addition growth factors may be required for the action of LH on the Leydig cell for its maximum function. The development of vaccines against growth factors which positively affect Leydig cell function could conceivably serve as a tool for the control of male reproduction in overpopulated countries. Further vigorous investigations of the disruption of growth factor receptor genes and their binding proteins by biological and chemical means would add to our understanding of the role of growth factors in control of male reproduction.

Finally, recent studies have indicated that there is a relationship between somatotropic signaling and longevity *(195,196)*. It has been suggested that reduction/impairment in GH/IGF-I secretion extends life span in animals and possibly also in human. In mutant mice, reductions in insulin and IGF-I signaling pathways and their impact on the hypothalamic–pituitary–gonadal system *(133,195,196)* indicate that delayed reproductive development and reduced fertility of these animals could be contributing to their longevity. It is possible that there might be a trade-off between longevity and fecundity.

There is limited information from studies of untreated patients with multiple pituitary hormone deficiency because of *Prop-1* gene mutation, and in Laron syndrome patients with isolated IGF-I deficiency caused by deletions or mutations of the *GHR* gene, indicating that some of the individuals can attain very advanced age *(197)*. In contrast, reduced longevity was reported in some untreated patients, who had isolated growth hormone deficiency *(198)*. More research is needed to elucidate the role of GH/IGF-I in longevity in human.

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19 Insulin-Like Peptide 3 in Leydig Cells

Richard Ivell, PhD and Ross A. D. Bathgate, PhD

SUMMARY

Insulin-like peptide 3 (INSL3) is a novel member of the insulin-relaxin family of structurally related peptides, which has evolved by sequential duplication from a common ancestor. INSL3 is expressed in large amount by fetal and adult-type Leydig cells, once these have attained a mature phenotype. Experimental evidence suggests that the expression of INSL3 is not acutely regulated, but rather reflects the differentiation status of the Leydig cells. As such it is not regulated by the acute response of the hypothalamic–pituitary–gonadal axis, but only in a chronic context, where LH is promoting Leydig cell differentiation. INSL3 can be measured in the male circulation with values for the human ranging between 0.5 and 2.5 ng/mL, all of which appears to derive from the testis. INSL3 acts through a G protein-coupled receptor called RXFP2 (relaxin family peptide receptor 2; previously called LGR8), which appears to be expressed in multiple tissues, including germ cells, where INSL3 seems to act as a survival or antiapoptotic factor. However, the principal function for INSL3, is in the male fetus, during the first phase of testicular descent, where INSL3 from the fetal Leydig cells promotes the growth and expansion of the gubernaculum, retaining the embryonic testis in the inguinal region. New research implicates the involvement of INSL3 downregulation in xenobiotic-induced cryptorchidism, following exposure of the pregnant mother to environmental endocrine disruption. However, there is as yet little evidence for the involvement of INSL3 or its receptor in natural human cryptorchidism. *Note:* for convenience, all references to INSL3 or its receptor make use of the human gene nomenclature, even for rodents.

Key Words: Cryptorchidism; testicular descent; gubernaculum; INSL3; relaxin; LGR8; RXFP2.

INTRODUCTION, DISCOVERY, AND BACKGROUND

For many years Leydig cells were seen as steroid factories and little else. This image is now very different, in part because of the discovery that Leydig cells are also producers of a major new peptide hormone, insulin-like peptide 3 (INSL3), whose properties and functions are still being elucidated. INSL3, formerly referred to as either Ley-IL or RLF (relaxin-like factor), is a member of the insulin-relaxin family of peptide hormones, and like these appears to function as an A–B heterodimer, following cleavage of a connecting (C–) peptide from a common precursor polypeptide. It is most similar in structure to the hormone relaxin, and like relaxin, acts on a novel member of a new class of G protein-coupled receptor, called RXFP2 (also called LGR8 or Great).

cDNA encoding INSL3 was first discovered by Adham and colleagues *(1)* in a differential cloning project looking for testis-specific genes from the pig. Subsequently, sequences were identified from the human *(2,3)*, mouse *(4)*, rat *(5)*, marmoset monkey *(6)*, various ruminants *(7–10)*, dog *(11,12)*, and other mammalian species. In all cases, INSL3 appears to be a major secretory product of testicular Leydig cells in the male. INSL3 is also expressed in female tissues (e.g., ovarian theca cells (*[7,13,14]* or placenta *[15,16]*), but in lower amount, implying only local, paracrine functions.

Comprehensive searching of the genome databases has demonstrated definitively that INSL3 orthologs are not present in any submammalian species *(17)*. Phylogenetic analysis indicates that the *INSL3* gene evolved from the relaxin gene, which itself emerged as a result of duplication of the ancestral relaxin-3 gene (Fig. 1). Hence, it is now widely accepted that INSL3 is a member of the relaxin peptide family and likely evolved together with the appearance of mammalian viviparity and a scrotal testis.

STRUCTURE, SYNTHESIS, AND PROCESSING OF INSL3

The structure of the INSL3 preprohormone conforms to the other members of the insulin/relaxin peptide

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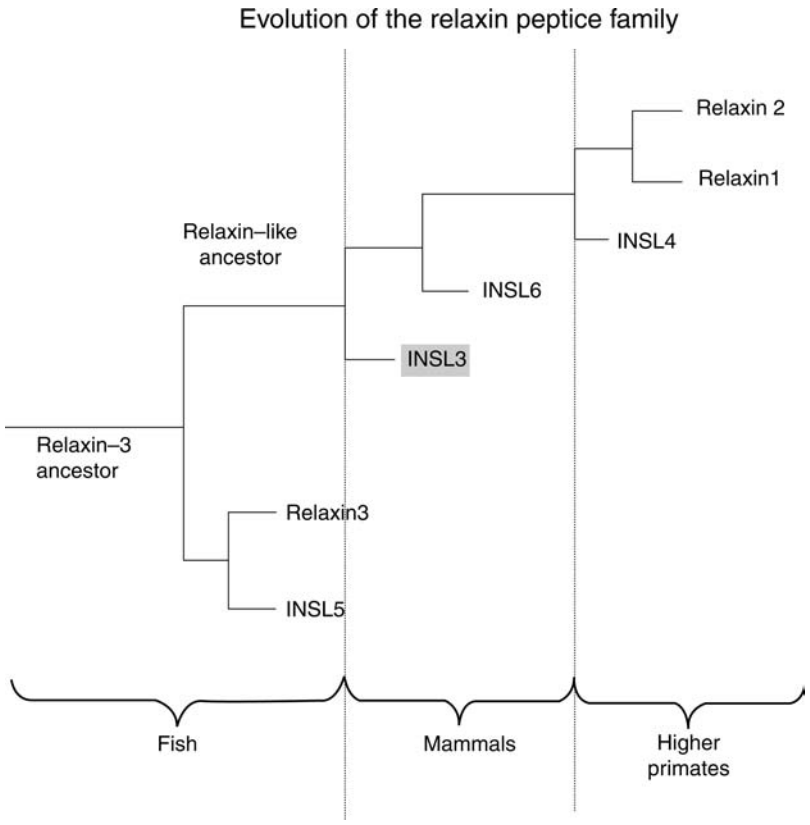


Fig. 1. Evolutionary relationships among the relaxin family of peptides highlighting that the *INSL3* gene evolved from a duplication of the ancestral relaxin gene in mammals. The consensus tree is based on previously published data *(17)*.

superfamily with a classic signal peptide sequence followed by the B/C/A domain peptide structure. The sequences of all known INSL3 preprohormones derived either by cDNA cloning or genomic database searching are aligned in Fig. 2. It is presumed that the C domain is removed before secretion of the native peptide, which consists of a B/A heterodimer with two interchain and one intrachain disulphide bond in the A chain. The junctions of the B/C and C/A chain are predicted either by alignment to other family members or by the presence of dibasic residues, which can be cleaved by proconvertase enzymes. A native INSL3 peptide has only been isolated from one species, the bovine *(18)*. This peptide, which was isolated from the bovine testis, is an A/B heterodimer, but has a longer B chain (seven amino acids) than predicted. However, this peptide may not reflect the structure of all INSL3 peptides, as the bovine sequence lacks dibasic residues at the end of the predicted B chain like those present in other species including human. Furthermore, it is possible that the peptide is further processed in the plasma to yield a shorter B chain. Importantly, a synthetic bovine peptide with a shorter B chain showed a higher affinity for INSL3 receptors than the peptide extracted from the testis with the longer B chain *(18)*.

There is considerable evidence that INSL3 peptides do conform to the predicted structure and circulate in the plasma. Synthetic human, rat, sheep, and bovine peptides based on these predictions have been demonstrated to have high-affinity for both the native and recombinant INSL3 receptor. Furthermore, assays of plasma levels of the INSL3 peptide use antibodies raised against the processed form of the peptide *(19–21)* and thus, predispose that the native peptides circulating in the plasma are likely to be in the predicted A/B heterodimeric form.

Alignment of the known INSL3 sequences demonstrates that there is large variability in the C chain whereas the A and B chains are highly conserved (Fig. 2). Structure/function studies have shown that the highly conserved motif –GGPRW- at the C-terminus of the B chain is a major determinant of INSL3 activity *(22)*. The tryptophan residue (W) likely interacts directly with the receptor whereas the other amino acids are necessary for the correct orientation of this residue. More recent studies have shown that N-terminal truncations of

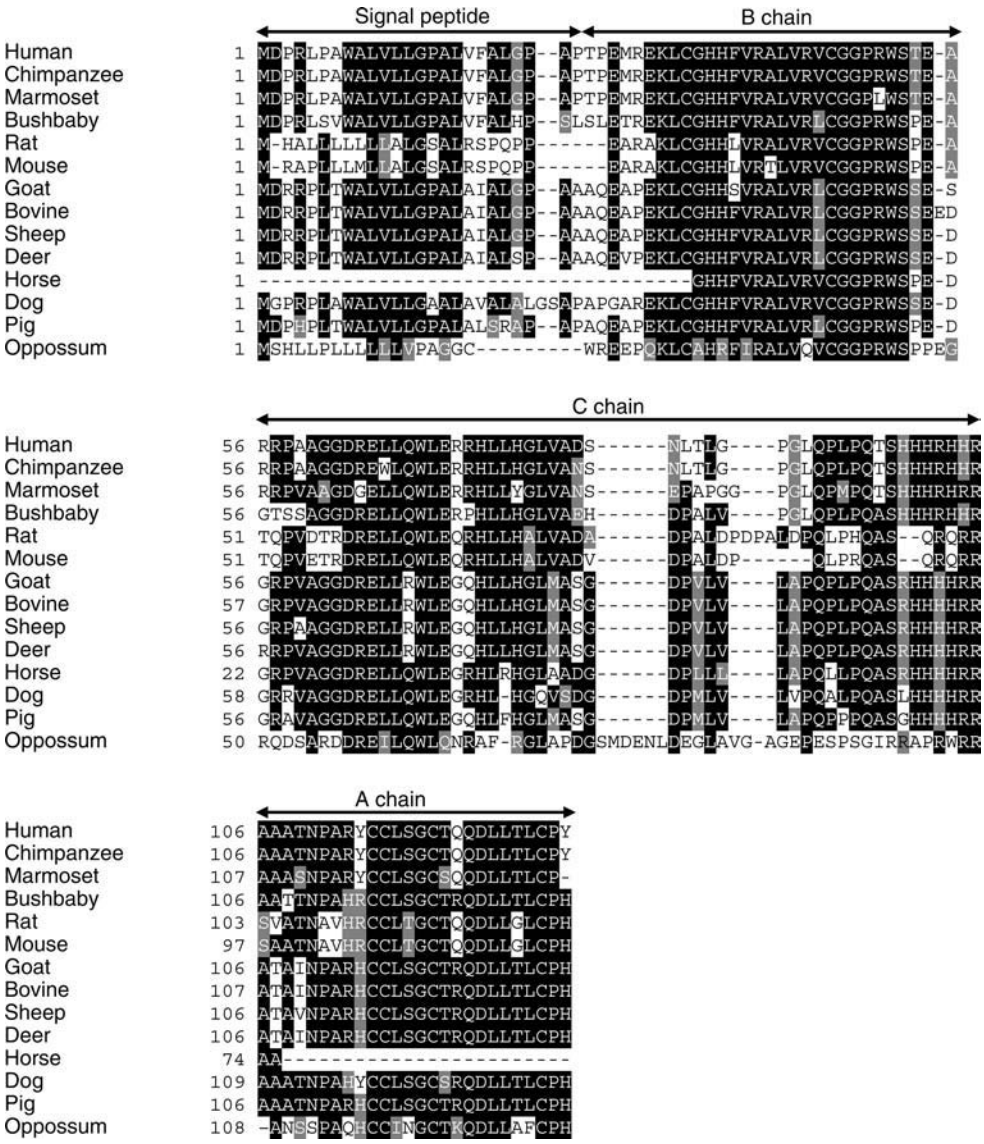


Fig. 2. Multiple alignment of all the known prepro-INSL3 sequences, including A-, B-, and C-chains, developed using the ClustalW algorithm. Human *(2)*, marmoset *(6)*, bushbaby *(73)*, rat *(5)*, mouse *(4)*, goat *(74)*, bovine *(7)*, sheep *(8)*, deer *(9)*, horse-partial *(49)*, dog *(12)*, and pig *(1)* are derived from published sequences. Chimpanzee and Oppossum sequences were identified from genome sequences in Ensembl (http://www.ensembl.org/) using tBlastN alignments with the human INSL3 sequence.

the A-chain do not affect binding of the peptide, but result in loss of bioactivity. Hence, the A-chain seems not to be involved in receptor binding, but is necessary for activation *(23)*.

EXPRESSION OF *INSL3*

Expression of the *INSL3* gene in Leydig cells correlates closely with the development of a differentiated phenotype. In wild-type rodents, where most studies have been carried out, *INSL3* mRNA expression is maximal in mature adult-type Leydig cells *(4,5,24)*. It is also expressed highly in mature fetal Leydig cells *(24,25)*. In the intermediate period between days 5 and 15 (for rats or mice), when immature Leydig cells predominate in the interstitium, *INSL3* mRNA is expressed at low level only. In general, INSL3 protein expression follows that for the mRNA, with first appearance of INSL3 immunoreactivity in the mouse between postnatal days 16 and 18 and in the rat at around day 20 *(5,24)*. The situation for INSL3 protein in fetal Leydig cells is more complicated. Antibodies, which work well in the adult testis, appear to be less effective in the fetal testis *(26)*. One explanation for this might be that INSL3 protein is expressed in the constitutive pathway,

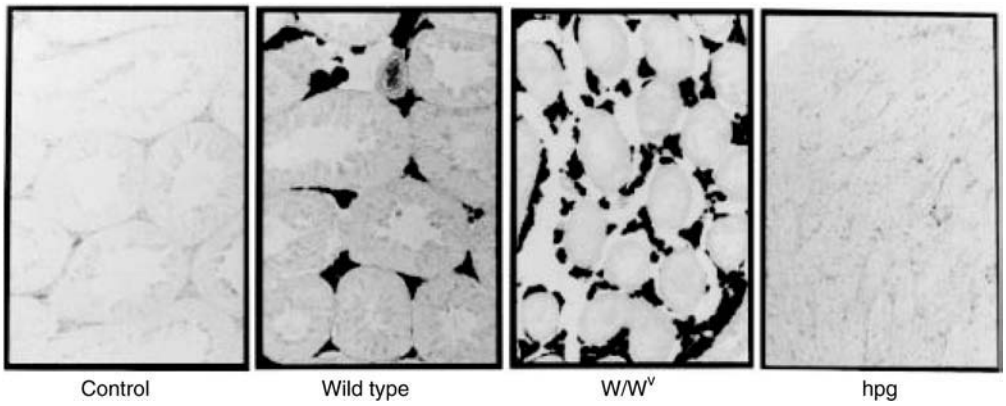


Fig. 3. *In situ* mRNA hybridization for *INSL3* transcripts in testis sections from wild-type, and from *w/w^v* and *hpg* mutant mice. The *w/w^v* mice owe their azoospermia to a mutation in the *c-kit* gene, but are otherwise endocrinologically adult. The *hpg* mice lack a functional HPG axis, and are hence arrested in a prepubertal state. The control (left panel) is of a wild-type testis subjected to *in situ* RNA hybridization using a sense-stranded probe. From ref. *24* with permission. Copyright © 1998 The Endocrine Society.

particularly in fetal Leydig cells, with protein being secreted from the cells as soon as it is made. In mature adult-type Leydig cells, immunohistochemistry suggests the subcellular localization of INSL3 epitopes within what is probably the Golgi complex, suggesting some form of storage before release *(24,25)*. More research is needed to examine these aspects of INSL3 synthesis, processing, and release.

Factors, which influence the differentiation of Leydig cells also influence the quantitative expression of INSL3. One of the best examples of this is the *hpg* (hypogonadal) mouse. These mice lack LH because of a deletion within the GnRH gene, and thus, have immature testes equivalent to those of a prepubertal mouse (Fig. 3). These testes express none or only very low levels of *INSL3* mRNA and protein *(24)*. Daily injections of human choriogonadotropin (hCG) to induce puberty and Leydig cell differentiation, lead to the maturation of the testes and to a marked upregulation of INSL3 protein and mRNA expression *(24)*. It is interesting to note that the fetal Leydig cell population of these *hpg* mice do express normal high levels of INSL3 (Fig. 4), concomitant with the LH-independence of fetal Leydig cell differentiation *(24)*. Seasonal breeding animals, such as the Djungarian hamster *(27)* or the roe deer *(10)*, show a similar expression pattern, with very low or no expression in the Leydig cells of the involuted testis, and increased expression as the testes mature during the breeding season.

Recently, immunoassays have been developed to measure INSL3 protein in human peripheral blood *(20,21,28)*. In men, the normal range for INSL3 in serum is between 0.5 and 2.5 ng/mL. In anorchid men and women, INSL3 is close to or lower than the level of detection, showing that in men the testis is the sole source of circulating INSL3, a fact reinforced by high levels of INSL3 (>20 ng/mL) measured in spermatic vein blood *(21)*. Of particular interest are measurements carried out on hypogonadotropic hypogonadal men or men whose hypothalamo–pituitary–gonadal (HPG) axis has been suppressed by contraceptive application of androgens. In these men, acute application of hCG (<96 h) has no effect on peripheral INSL3 levels, although causing a marked increase in testosterone *(21)*. However, longer gonadotropin treatment leads to a differentiation of Leydig cells and hence, to an upregulation of circulating INSL3 *(20)*.

In studies to assess factors, which might influence INSL3 expression in Leydig cell cultures, we have been unable to identify any effectors able to either increase or decrease *INSL3* mRNA expression using both mouse primary cell cultures, as well as the mouse MA-10 Leydig tumor cell line *(24,26)*. These included hCG, db-cAMP, atrionutriuretic peptide, phorbol myristate acetate, testosterone, estradiol, dexamethasone, IGF1, tumor necrosis factor-α, and transforming growth factor-β *(26)*. Together these studies reinforce the viewpoint that *INSL3* gene expression is constitutive once the mature phenotype is attained, and only factors, which can influence the differentiation status of Leydig cells are able to influence the level of INSL3 expression. This is an important finding, because it means that measurement of INSL3 in peripheral serum is an indicator for Leydig cell differentiation status, independent of other factors, including the HPG axis.

It has been shown that INSL3 is downregulated in aging rat Leydig cells *(29)*, and more recently, that human peripheral INSL3 is also decreased in older men

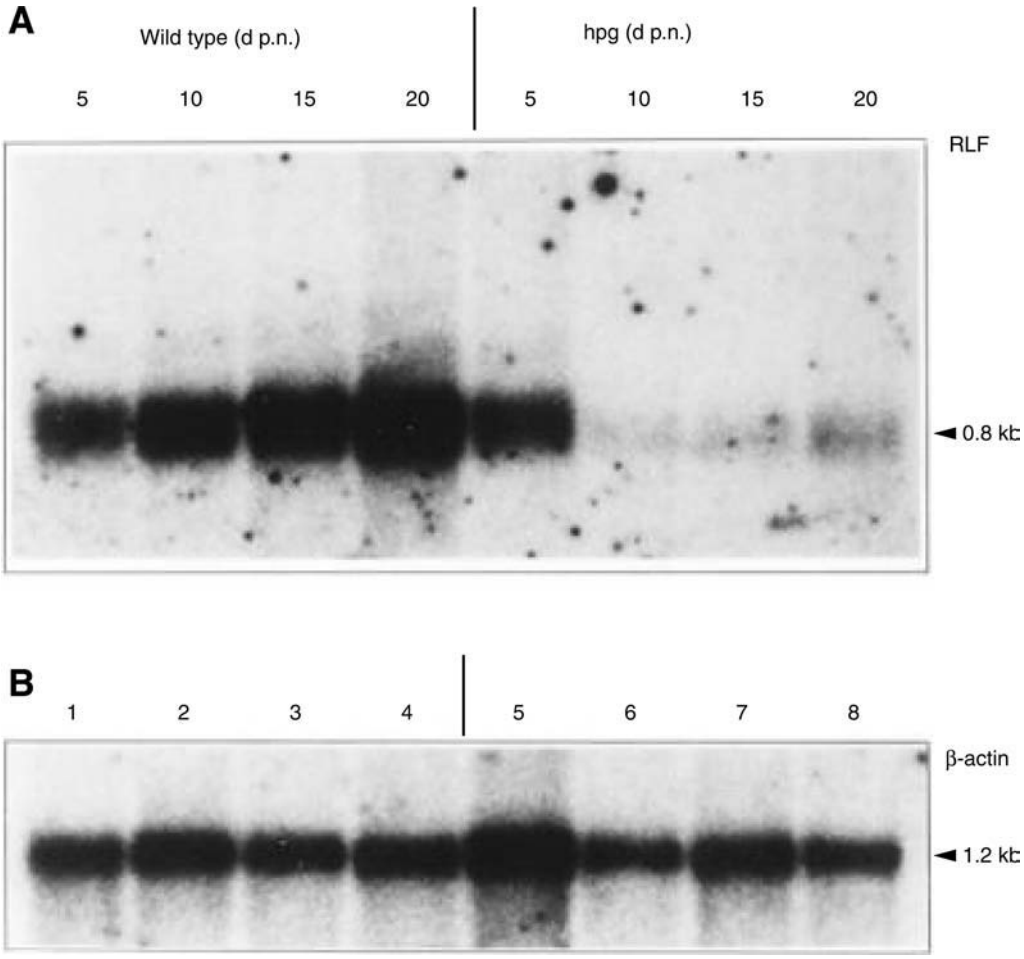


Fig. 4. Northern hybridization of *INSL3* mRNA from the testes of wild-type and from *hpg* mice at different postnatal ages. Note the apparently normal signal in the RNA sample from pnd 5 of the hpg mouse. Here the testes contain fetal Leydig cells whose mature phenotype is attained independently of LH. From ref. *24* with permission. Copyright © 1998 The Endocrine Society.

(29a). INSL3 also becomes downregulated in Leydig cell tumors *(30)*, where presumably transformation and the resumption of the cell cycle lead to a dedifferentiation of the Leydig cell phenotype. It is to be noted that rodent tumor Leydig cell lines in general, express less *INSL3* mRNA than freshly prepared primary Leydig cells (personal observations). In the context of human testicular pathology, immunohistochemistry was unable to show any differences of *INSL3* expression across a range of spermatogenic phenotypes *(3)*, although, application of the new INSL3 immunoassay did show a reduction of peripheral INSL3 in Klinefelter patients *(21)*. Whereas in healthy men, peripheral INSL3 appears to derive almost exclusively from the Leydig cells of the testis, it is possible that in some pathological situations INSL3 might be contributed by other organs. For example, it has been shown that certain Leydig-like tumors of the adrenal gland begin to express INSL3, unlike the normal human adrenal gland *(31)*, and it has recently been shown that the prostate and thyroid glands may also be a source of INSL3 *(32,33)*.

As discussed earlier, it is assumed that the form in which INSL3 is secreted is that of a processed A–B heterodimer, analogous to that of insulin and relaxin. To date the only evidence for this is the work of Bullesbach and Schwabe *(18)*, where the 6 kDa A/B heterodimer was extracted from the bovine testis and subjected to MS sequencing. However, the reverse-phase methodology used excluded larger sized polypeptides from analysis. There are a few reports using Western blotting of testis and other organ extracts, where an immunoreactive protein band is seen at between 10 and 18 kDa (e.g., ref. *16*), supposedly corresponding to the unprocessed precursor, which has retained the C domain. Given the immunohistochemical observation of a focal concentration of staining in a perinuclear, Golgi-like region of the Leydig cell, this would imply that most INSL3 inside the adult testis is

probably unprocessed, and thus should conform to the larger precursor structure. However, it is to be noted that where attempts have been made to produce INSL3 recombinantly, for example, in insect cells *(6)*, this also appears to be secreted from the cell in an unprocessed form. Thus, at present it is not possible to state whether the major circulating form of INSL3 is the cleaved heterodimer or the unprocessed proform; both are likely to be immunoreactive in the assays used, and both are likely to be bioactive.

THE *INSL3* GENE AND ITS VARIANTS

Like the other genes of the insulin-IGF-relaxin superfamily, INSL3 is also encoded on two exons, with an intron inserted into the region encoding the C domain. In the human it is located on chromosome 19p13.2, in the mouse on chromosome 8B3.3. In all mammalian genomes so far examined, the *INSL3* gene is in very close proximity, or overlapping with the gene for the tyrosine kinase JAK3 *(34)*. In the mouse and rat, the last exon of *JAK3* is coincident with exon 2 of *INSL3*, although, different splice sites are used; exon 1 of the rodent *INSL3* gene is located within the last intron of the *JAK3* gene. It would appear that there is a degree of promiscuity about splice donor and acceptor sites in this genomic region. Besides the different splicing pattern for *JAK3*, cDNA cloning of *INSL3* transcripts from different species has revealed the existence of several *INSL3* splice variants *(6,26)*. These mostly represent extended exon 1 forms, resulting in the introduction of a novel stop codon and a truncated reading-frame, together encoding a polypeptide comprising an extended B-domain only. Whether such peptides are synthesized in vivo, and whether these are functionally relevant is still unknown.

The immediate upstream promoter region of the *INSL3* gene has been studied for the mouse *(35,36)*, rat *(26)*, and bovine (unpublished observations). Although, transgenic studies have not yet been used to define regions of cell type and temporal specificity within the promoter, transfection studies have shown that a relatively short region (<200 bp) is sufficient for high expression in various Leydig tumor cell lines *(26,34–36)*. Interestingly, this region contains three sites capable of binding the transcription factor steroidogenic factor (SF)-1 in all of the species analysed. Comparing the rat and mouse promoters, it would appear that the three SF-1 sites are not used to a similar extent in the two species, with the proximal site being more important for the mouse gene, whereas the distal site appears to have this role in the rat *(26)*. It should be stressed that the in vitro assays used are very artificial, and need not reflect the in vivo situation. Indeed, a recent study suggests that not SF-1, but other transcription factors, such as NR4A1 (Nur77), might be more relevant in the context of Leydig cell expression *(37)*. As for other SF-1 regulated genes, it has also been shown in transfection experiments that the activating effect of SF-1 on the mouse *INSL3* promoter can be inhibited by coexpression of the negative factor DAX1 *(36)*.

INSL3 RECEPTORS (RXFP2/LGR8)

The receptor for INSL3 is the leucine-rich repeat containing G protein-coupled receptor 8 (LGR8). Receptors of this family include the receptors for luteinizing hormone, follicle stimulating hormone, and thyroid stimulation hormone, three orphan receptors LGR4-6 and the relaxin receptor LGR7 *(38)*. All the receptors are characterized by a large extracellular domain with multiple leucine-rich repeat domains. LGR7 and LGR8 form a subclass (subgroup C) of the LGR family as a result of their close sequence homology and the presence of an N-terminal low-density lipoprotein class A module. LGR7 and LGR8 are now classified as relaxin family peptide receptors, RXFP1 and RXFP2, respectively *(39)*.

The INSL3 receptor was first identified because of the similarities in phenotype of mice lacking the INSL3 gene and mice lacking a 550 kb region of chromosome 3, which contains the *RXFP2* gene *(40)*. Subsequent studies using a specific RXFP2 KO mouse confirmed that the phenotype is identical to that of the INSL3 KO *(41)*. Pharmacological studies on recombinant receptors have demonstrated that synthetic INSL3 is able to activate the human *(42)*, rat *(43)*, and mouse (Bathgate, unpublished) RXFP2 receptors with subnanomolar affinity. Furthermore, synthetic INSL3 is able to induce the growth of gubernacular cells in organ culture *(44)* and in cultured fetal gubernaculum cells in vitro *(42)*, acting through LGR8 receptors expressed in the gubernaculum. Relaxin is also a ligand of the RXFP2 receptor, although there are marked species differences in the activities of relaxin peptides on the receptor *(39)*. Importantly, mouse and rat relaxin will not activate the RXFP2 receptor, hence relaxin is not a ligand of the RXFP2 receptor in rodents. Indeed, cross-breeds of INSL3-overexpressing mice, RXFP2-, and RXFP1-receptor KO mice have firmly established that RXFP2 is the only receptor for INSL3 in mice *(45,46)*. However, it is still possible that in other species, including human, relaxin is a ligand for the RXFP2 receptor in vivo.

RXFP2 receptor expression has been demonstrated in numerous tissues in both males and females, although, most studies have only identified sites of mRNA expression. Numerous nonfunctional splice variants of the RXFP2 receptor have recently been characterized *(47)*. Hence, sites of expression demonstrated by reverse transcriptase polymerase chain reaction (RT-PCR), *in situ* hybridization, and even Northern blotting do not necessarily demonstrate the presence of functional RXFP2 receptors. The gubernaculum is clearly a site of RXFP2 receptor expression as isolated gubernacular cells respond to INSL3 stimulation as outlined earlier. The testis is a site of RXFP2 receptor mRNA expression in the rat *(43,48,48a)*, mouse *(40,48a)*, roe deer *(10)*, horse *(49)* and human *(20,38)*. In rats, functional RXFP2 receptors appear to be present on testicular germ cells and are associated with the inhibition of apoptosis *(48)*, and both gene expression and ligand binding are demonstrated for Leydig cells *(48a)*. In the rat ovary, functional RXFP2 receptors on oocytes have been implicated in oocyte maturation *(48)*. The kidney is also a site of RXFP2 expression in the human *(20,38)* and rat *(50)*. In the rat RXFP2 mRNA is localized on mesangial cells in the glomerulus and INSL3 is able to inhibit the proliferation of cultured mesangial cells *(50)*. The functional significance of this observation is unknown. Similarly, RXFP2 expression and INSL3 action are reported for prostate carcinoma cells *(33)*, although their function is unclear. RXFP2 mRNA is expressed in the human *(38,51)* and porcine *(52)* uterus and INSL3-binding sites have been demonstrated on mouse uterine membrane extracts *(53)*. RXFP2 mRNA is also expressed in the rat brain *(54)* and INSL3-binding sites have been previously demonstrated in mouse brain membrane extracts *(53)*. However, as expression of INSL3 peptide has not been demonstrated in the brain, and it is unlikely that the peptide will cross the blood–brain barrier, the functional significance of this expression is unknown.

There is currently very little known about the mechanisms of INSL3-binding to its receptor or the signaling pathways utilized to induce its cellular actions. Current evidence suggests that INSL3 binds to a primary site in the leucine-rich repeats of the receptor ectodomain as well as a secondary site in the transmembrane domains *(55,56)*. Subsequent signaling of the ligand–receptor complex is directed by the low-density lipoprotein class A module *(39)*. Recombinant RXFP2 receptors expressed in mammalian cells respond to INSL3 stimulation with dose dependent increases in cAMP *(38,43)*. Stimulation of primary rat gubernacular cells in culture by INSL3 also results in dose-dependent cAMP stimulation *(42)*, as is also true for prostate carcinoma cells *(33)*. Therefore, it is likely that coupling to stimulatory G proteins to induce increases in cAMP is an important mediator of the cellular actions of INSL3. However, INSL3 appears to induce its effects on testicular germ cells and oocytes through coupling to inhibitory G proteins, and hence decreased cellular cAMP *(48)*.

FUNCTIONS OF INSL3

To date, much of the information that we have about a function for INSL3 comes from studies on mutant mice, where either the gene for INSL3 *(57,58)* or for its receptor *(46)* have been ablated, or where INSL3 has been constitutively expressed from a strong promoter also in females *(59,60)*. The knockout mice consistently show that the principal function of INSL3 is to promote the growth and development in the fetus of the gubernacular ligament, which connects the testes to the inguinal region. Thus, by inducing gubernacular thickening, the fetal testes, which initially are in a perirenal position, are effectively retained in the inguinal region, whereas the remainder of the body, including the kidneys, grow dorsally, thus contributing to the first phase of testicular "descent." This occurs in the middle of the second trimester of pregnancy in most species.

Whereas INSL3 is essential for this process, at least in rodents, it appears that both androgens and antimullerian hormone may have a synergistic effect *(44)*. As a result, newborn rodents lacking INSL3 are cryptorchid, and unless surgically corrected, secondary effects of the cryptorchidism (influence of body temperature on the abdominal testes) will lead to defects in spermatogenesis. In human, cryptorchidism is associated with higher risks of male infertility and testicular cancer *(61)*. Recently, it appears that INSL3 may also be involved directly in spermatogenesis in the adult. Injection of INSL3 into the testes of adult rats, which had been treated with a GnRH antagonist, appears to decrease the rate of germ cell apoptosis *(48)*. The authors suggest that INSL3, acting through the RXFP2 receptor on germ cells, may mediate the actions of LH on germ cell survival. Recent studies in rats using a specific RXFP2 receptor antagonist to block the action of endogenous INSL3 on germ cells, and subsequently to increase germ cell apoptosis, support this view *(61a)*. Hints at other possible functions for INSL3 have been gained by studies in female rodents. First, INSL3 gene ablation appears to be associated with a decrease in fertility, and increased cycle length, consequent on increased apoptosis of

follicles and corpora lutea *(58,62)*. This is supported by immunohistochemical studies in the cow, where INSL3 expression in follicles correlates negatively with atresia *(63)*. There is also evidence from studies in rats that INSL3 acts directly on oocytes to induce their maturation *(48)*. Secondly, a gain-of-function transgenic mouse overexpressing INSL3, also in the females, shows that the hormone can induce abdominal hernia, evidently weakening the muscle wall of the lower abdomen in the inguinal region *(59,60)*, besides forcing development of the gubernaculum and retention of the ovaries in an inguinal position.

There are likely to be other subtle functions for INSL3, which will only come to light after extensive experimentation. Now that we know that there is only one receptor for INSL3 in rodents, namely RXFP2 *(45)*, and that RXFP2 expression can be defined by RT-PCR, immunohistochemistry, and by knock-in of chromogen into gene-ablated mice, it is evident that RXFP2 is expressed in a wide range of tissues. To date, there is very little evidence for INSL3 function in these tissues. However, because INSL3 appears to be a relatively modern hormone in evolutionary terms, it is likely that many of these functions are to a degree redundant, with roles shared with other hormone systems. It should be emphasized here that all of the methodologies mentioned earlier to detect RXFP2 expression must be accompanied by extensive controls. It has recently been shown that the RXFP2 gene can be expressed in multiple splice variant forms *(47)*, of which only the full-length variant is likely to be functional in a conventional sense. Furthermore, it appears that some tissues express relatively large amounts, of splice variant forms, whose function is as yet unknown. Simple RT-PCR assays, immunohistochemistry using monotypic antibodies, or chromogen knock-in experiments are probably alone not sufficiently stringent to determine any kind of functionality for the LGR8 gene product detected.

INSL3 AND ENVIRONMENTAL ENDOCRINE DISRUPTION

Cryptorchidism in male offspring has been recognized for a number of decades as a common sequel to environmental endocrine disruption caused by predominantly estrogenic agents. Early studies on the effects of diethylstilbestrol on pregnant women *(64)* and rats *(65)*, as well as the later description of the testicular dysgenesis syndrome (TDS) *(66)* emphasize cryptorchidism as a key symptom of xenoestrogenic exposure. It was shown that there was a decrease in *INSL3* mRNA in fetal rat testes following diethylstilbestrol exposure of the mothers *(67,68)*. Since then, INSL3 production by fetal Leydig cells, at both mRNA and protein levels, has also been implicated in the cryptorchidism of male offspring from female rats treated with phthalates *(25,69,70)*. The mechanism by which these xenobiotics influence *INSL3* gene expression is not known. There are no classic estrogen response elements in the known *INSL3* gene promoter region, nor is the mechanism of action of phthalates fully understood. They may act through estrogen-independent pathways.

Following these experimental studies, there has been considerable research carried out to determine whether INSL3 is involved in human cryptorchidism, particularly in the context of TDS. Preliminary studies assessing INSL3 protein in cord bloods of newborn cryptorchid boys indicated no significant differences from a control group (Bay, Toppari, and Ivell, unpublished), though the relevant age of the transabdominal phase of testicular descent in humans would have been substantially earlier. There have been numerous attempts to find mutations in the *INSL3* and *RXFP2* genes, which associate significantly with cryptorchidism. To date, however, very few of the discovered nucleotide changes can be causally linked to a failure of testicular descent (reviewed in ref. *71*). Consequently, only two peptide and one receptor mutation are linked to disruptions in the INSL3/RXFP2 system. A nonsense mutation in the C peptide of pro-INSL3 (R49X) results in a nonfunctional peptide *(72)* and a proline to serine mutation in the B chain of INSL3 results in reduced activity of the INSL3 peptide *(45)*. This highly conserved proline residue has been previously demonstrated to be essential for the activity of the INSL3 peptide *(22)*. A proline to threonine substitution at position 222 in the fourth leucine-rich repeat of the RXFP2 ectodomain results in a receptor which does not respond to ligand in vitro *(41)*. These mutations are very rare, and cannot account for the high incidence of cryptorchidism found in the human population.

Some domestic species, such as dogs and horses, suffer high incidences of cryptorchidism, which may or may not be exacerbated by environmental influences. To date there is a single study to suggest that in horses cryptorchidism may be associated with a disruption of the INSL3/RXFP2 system *(49)*. Further more, taking advantage of the extensive breeding paradigms possible with domestic species will help to clarify the role of the INSL3/RXFP2 hormone system in this pathology.

OUTLOOK AND CONCLUSIONS

The discovery of substantial INSL3 production by both fetal and adult-type Leydig cells, thereby contributing to most if not all of the hormone circulating in the blood, together with its apparent independence of the HPG axis and other acute effectors, means that INSL3 is a new and important peripheral marker for Leydig cell status, and indirectly for testis status. To date, the relative lack of available reagents has severely limited clinical applications of these findings, but it is to be expected that new functions and aspects of INSL3 physiology will be quickly defined, with INSL3 forming an important cornerstone in our understanding of the fetal and adult-type Leydig cell and their pathologies.

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20 Neuronal Signaling Molecules and Leydig Cells

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SUMMARY

Innervation of the mammalian testis and the proximity of nerve fibers and Leydig cells are obvious anatomical bases for nerve-derived catecholamines and neuropeptides to affect Leydig cells. In addition, Leydig cells, as well as other somatic and germ cells, are producers of several types of neuronal signaling molecules, including γ-aminobutyric acid (GABA) and histamine. As Leydig cells express receptors for these signaling molecules, auto/paracrine effects must be assumed. Although, there still are only few functional studies, their results clearly indicate that steroidogenesis and Leydig cell proliferation and/or differentiation are under the influence of neuronal signaling molecules. The degree and the pattern of innervation, intratesticular production of signaling molecules as well as the responsiveness of Leydig cells are not fixed but show great plasticity. It is becoming clear that they all strongly depend on the functional and developmental state of the testis and the Leydig cells. Furthermore, changes of the density of the innervation recently observed in testes of infertile men as well as changes in intratesticular histamine sources, not only hint to a fundamental involvement of these as yet ill-recognized, regulatory systems in general Leydig cell physiology, but also to roles in pathology. Because many drugs used in humans for various reasons can interfere with the action of catecholamines, GABA, and histamine, the identification of these signaling systems in human testis is of as yet unexplored potential clinical relevance.

Key Words: Catecholamines; development; GABA; histamine; mast cell; neurotransmitter; steroidogenesis.

INTRODUCTION AND FOCUS OF THE ARTICLE

In addition to the endocrine system the nervous system directly (through innervation) or indirectly (e.g., through altering vascular tone) participates in the regulation of most organs. Presence of testicular nerves have long been described in most species (*see* refs. *1,2*) and consequently, questions about their function(s) and possible role in Leydig cells regulation are old topics (*see* ref. *1*). Here the focus is on the current state of knowledge of innervation of the mammalian testis in respect of Leydig cells. Recently recognized intratesticular sources of neuronal signaling molecules are also addressed. Accordingly, evidence for Leydig cell regulation by the major neurotransmitters of the testicular innervation, catecholamines, will be in the center of this article. A brief summary of potential involvement of some neuropeptides and of acetylcholine and the emerging role of two locally produced signaling molecules, GABA, and histamine will be discussed. These are produced in the testis by Leydig cells (GABA) or mast cells (histamine). Because of space limitations, neither a number of intratesticular peptides, which have been reported to be present in nerve fibers and/or testicular cells (including opiates, somatostatin, oxytocin, galanin, and tachykinines; *see* ref. *3* and others for details), will not be discussed will be the complex serotonin–corticotropin-releasing factor system of the testis (*see* refs. *4,5*) addressed.

TESTICULAR SOURCES OF NEURONAL SIGNALING MOLECULES

Testicular Innervation

Light and electron microscopic studies as well as histochemical and newer immunohistochemical investigations have been performed in various animals (*1,2,6–17, see* ref. *18*) for more references. Despite the different technical approaches that were used, these studies have led to the overall view that the nerve fibers of the testis are mainly, if not exclusively, sympathetic in nature (*see* refs. in reviews *1,2,18–20*). They share this feature with the ovarian nerve fibers, but compared

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with the female gonad, testicular nerve fibers are much less frequently encountered *(2)*. Furthermore, strong species differences (e.g., almost complete lack of nerve fibers in the rat; refs. *21,22*), as well as differences in respect of developmental stages exist. Unfortunately, the lack of systematic studies using different complementary techniques throughout ontogeny, makes it difficult to conclude about presence and importance of many of the neuronal signaling molecules reported in general. For instance, cats *(7)* are reported to have rather dense innervation and this is evidenced by immunolocalization of neuronal markers, including protein gene product 9.5, neuropeptide Y (NPY), C-terminal peptide of neuropeptide Y, galanin, vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP), and substance P. A comparable array of antibodies for immunohistochemistry has not been used in other studies, with the consequence that the data reported in this study cannot be generalized. The same is true for Leu-enkephalin, somatostatin (e.g., in pig ref. *6*), or metenkephalin-containing nerves *(23)*, examined only by few investigators, and which like most of the aforementioned will therefore not be further discussed.

Testicular nerve fibers, when present, reveal typical ultrastructural signs (e.g., dense core granules presumably containing the typical neurotransmitter of the postganglionic neuron of the sympathetic chain, norepinephrine). They also contain enzymes responsible for catecholamine synthesis, tyrosine hydroxylase (TH), dopamine β-hydroxylase (DBH), and neuropeptides (as mentioned earlier, mainly VIP and NPY (*2,7,17,22,24; see* refs. *2,22* for references; Fig. 1). Indeed, there is strong consensus for the presence of TH (and thus norepinephrine, respectively), VIP and NPY and these signaling molecules will consequently be discussed (*see* "Neuropeptides [VIP, NPY, and CGRP] and ACh").

Evidence for presence of acetylcholine (ACh), the prototype postganglionic neurotransmitter of the parasympathetic nervous system in testis, is in comparison rather weak. ACh was, for instance, suggested by translucent clear granules in varicosities of testicular nerve fibers in electron microscopic studies (*see* refs. *16,18*). Measurements of intratesticular ACh levels are, to the author's knowledge, not published and it is not described whether the typical ACh-synthesizing enzyme (choline acetyltransferase; [CHAT]) is present in testicular nerve fibers. Indeed, our unpublished results do not support such a notion. Thus, although VIP, for instance, may be colocalized with ACh in parasympathetic nerve fibers (*see* ref. *25*), it remains to be clearly shown, whether and, if so, to what extend cholinergic, parasympathetic innervation of the testis exists *(2)*.

The nerves fibers, which travel with the arteries to the testes (superior spermatic nerves), or follow the ductus deferens (inferior spermatic nerves), are presumably efferent and/or afferent in nature, but details remain unknown. Only a few newer reports address this issue and interpret CGRP-positive fibers as afferent fibers *(9,12)*. These fibers are found much less often than those containing TH, for example, and CGRP-positive fibers are mainly confined to the tunica albuginea. When detected within the testes, they were noted to be not associated with intratesticular blood vessels in the interstitial area *(12)*, whereas in contrast they are clearly associated with extratesticular blood vessels in rats *(26)*.

Several different retrograde tracing methods have been used to decipher the neuronal connections of the testis in the rat and have provided insights into a multisynaptical pathway, by which these fibers are linked to the brain *(22,27,28)*. Labeled neuronal cell bodies were localized in the major pelvic and accessory ganglia (presumably representing the second, postganglionic neuron of the sympathetic chain), and spinal cord (T10-L1; L5-S1, representing the the first, preganglionic neuron). These preganganglionic neurons are furthermore connected through a multisynaptic neuronal chain to many neurons of the brainstem, hypothalamus, and even to telencephalic areas *(27,28)*.

All intratesticular nerve fibers are restricted to the interstitial compartment, where most appear to run along the arteries and arterioles before they branch off within interstitial cells and end at the wall of seminiferous tubules *(18,29–31)* (Fig. 1). Varicosities, i.e., swellings occurring in testicular nerve fibers, contain vesicles with stored neurotransmitters. Upon release these signaling molecules diffuse within the interstitial fluid to reach nearby target cells. These targets can be defined by expression of respective receptors and must be closeby, for sufficently high concentrations of signaling molecules to reach and activate the receptors. Cellular targets may include the smooth muscle cells of blood vessels (regulating blood flow; *see* ref. *32*). Other targets include myoid cells of the seminiferous tubule wall *(18,31)*, any other interstitial cells (e.g., immune cells, including mast cells *[33]*), but in particular Leydig cells. This type of "indirect innervation" (*see* ref. *34*) is the principal mechanism by which nerve fiber-derived neurotransmitters could interact with Leydig cells. In the human testis, Leydig cells were observed 150 nm–1 μm (*see* ref. *15*) apart from terminals or varicosities. As density of innervation may vary within the testis, regional differences may result in heterogeneity of Leydig cell responsiveness

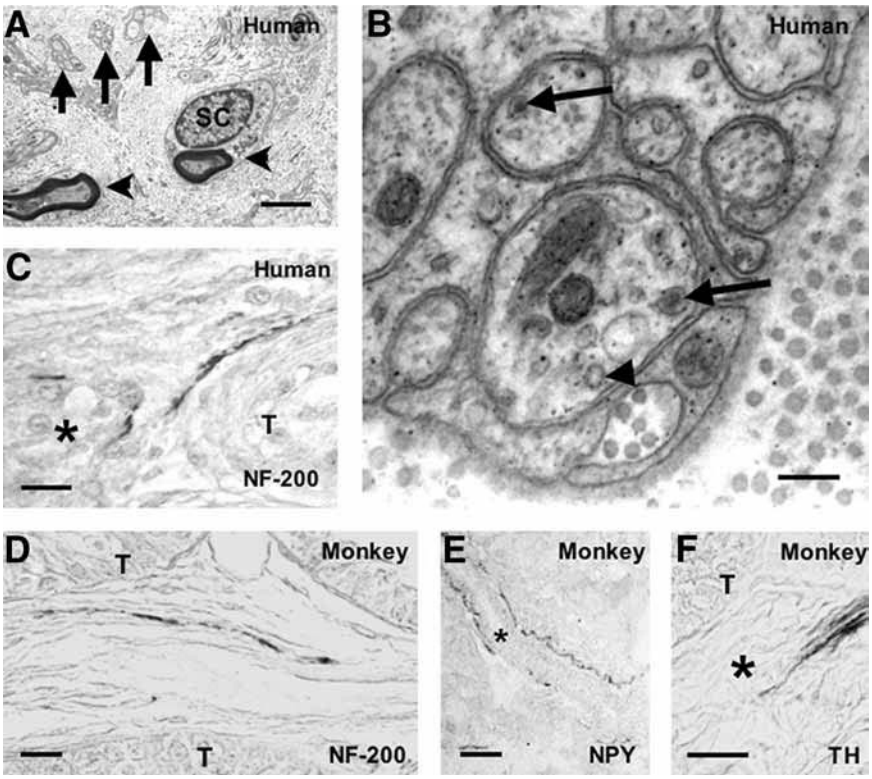


Fig. 1. Phenotypes of testicular nerve fibers in human and monkey testis. **(A)** An electron micrograph depicts a cross-section through several testicular nerve fibers in a human testis. Note a nucleus of a Schwann cell (SC) associated with a small myelinated axon (arrowhead), another myelinated axon (arrowhead) and several unmyelinated nerve fibers (arrows). Bar approx 3 µm. **(B)** A cross-section through a varicosity in the course of an unmyelinated nerve fiber in a human testis, with several dense core vesicles (arrows) is shown in higher magnification. These vesicles are the morphological correlate of catecholamine- and neuropeptide-containing vesicles. Note that also clear vesicles with electron-translucent content are present (arrowhead). This may represent an artifact (loss of electron dense material durings procesing of the sample) or may indicate presence of other neurotransmitters, for example ACh. The concept of "indirect innervation" is based on the release of the contents of these vesicles into the interstitial fluid, in which through diffusion it can reach the neighboring Leydig cells. Bar approx 200 nm. **(C)** Immunohistochemical staining of neurofilament 200 (NF-200), clearly depicting interstitial nerve fibers in proximity of Leydig cells (asteriks) and the peritubular region of a seminiferous tubule (T). Bar approx 10 µm. **(D–F)** Immunohistochemical staining of NF-200 (D), NPY (E; close to a blood vessel; asteriks) and the rate-limiting enzyme for catecholamine biosynthesis, tyrosine hydroxylase (TH; F) in rhesus moneky testes. Leydig cells (asteriks), seminiferous tubule (T). Bars approx 10 µm.

and, in general, in differences in respect of control exerted by released neurotransmitters (*see* refs. *20,35*).

In man, cat, guinea pig *(18,20,32,36)*, as well as in dog and rhesus monkey (Mayerhofer, unpublished) another type of innervation, resembling more a typical synaptic contact, occurs between nerve fibers and Leydig cells. Studies by Prince *(15)*, showed how Leydig cells in the human testis receive direct, selective innervation, with noradrenergic nerve terminals/varicosities being only 20 nm apart from the cell membrane of Leydig cells. It is unknown whether all Leydig cells within the testis would receive this type of direct innervation or whether, to what extent regional or Leydig cell-specific differences may exist.

Plasticity During Development and Pathology

The major signaling molecule of sympathetic nerve fibers of the testis, norepinephrine, has been measured in testicular homogenate and results indicate changes depending on age and functional stage. Testicular catecholamines (and/or histamine; *see* "Histamine") levels are higher in prepubertal rats and golden hamster than in adults *(37–40)*. Likewise, we observed higher testicular concentrations of norepinephrine in the prepubertal male gonad of the rhesus monkey as compared with the adult *(41)*. Thus immature stages are associated with higher levels, a fact which relates to function.

The main reasons for these changes are likely changes in innervation and/or intratesticular sources,

but alterations in biosynthetic activity cannot be ruled out either. Only the first mentioned possibility is documented, for example, in the boar, in which a dramatic loss of interstitial nerve fibers is associated with sexual maturation *(13)*. A similar tendency was reported in the bovine *(9)* and cervine species *(12)*. Furthermore, there is the possibility of seasonal changes, as suggested by increased neuronal elements in reproductively quiescent cervine testes, as compared to active testes with active spermatogenesis (*see* ref. *12*). In human testis Nistal et al. *(18)* reported many varicosities of nerve fibers adjacent to myoid cells in infants. In more detailed study, Prince *(15)* described dramatic increases in the number of nerve fibers (as well as of differentiated Schwann cells and terminals), from neonatal to the prepubertal stage. Evidence for a comparable plasticity of testicular innervation is provided by own studies in the rhesus monkey, which have used nerve specifc immunohistochemical markers (e.g., neurofilament-200 [NF-200]) and morphometric approaches to document these changes *(17,24)*. The intratesticular levels of norepinephrine and of TH-immunoreactive fibers decreased when animals reached adulthood and, as mentioned, this is mirrored by intratesticular norepinephrine concentrations. The reason for the "loss" of TH fibers in face of rather constant NF-200-positive fibers, remains to be shown, but a developmental switch in their neurotransmitter phenotype (*see* ref. *25*) cannot be ruled out.

Interestingly, in addition to these physiological changes, the density of the innervation in the testes of infertile men with deranged spermatogenesis is increased *(17,18)*. Again electron microscopy, but mainly immunohistochemistry (visualizing NF-200 and others) and morphometric approaches have lead to these results in testicular biopsies from men with different spermatogenesis problems (e.g., germ cell arrest and Sertoli cell only syndrome) (Fig. 2). Although, only small numbers of biopsies were evaluated from a heterogenous population of patients with idiopathic infertility, the observed increases in nerve fibers were common and clearly evident in practically all samples, thus, arguing for a "real" increase.

Clearly, at present time the causes for the observed plasticity of testicular innervation depending on development or pathological states are known. It can be speculated that neurotrophic factors, which are known to be present in testes (*see* discussion in ref. *41*; and for instance in ref. *25*) may be involved and that their availability may regulate the degree of innervation. Could the observed change also indicate involvement of neuronal signaling molecules in testicular development, with many fibers and high levels of signaling molecules

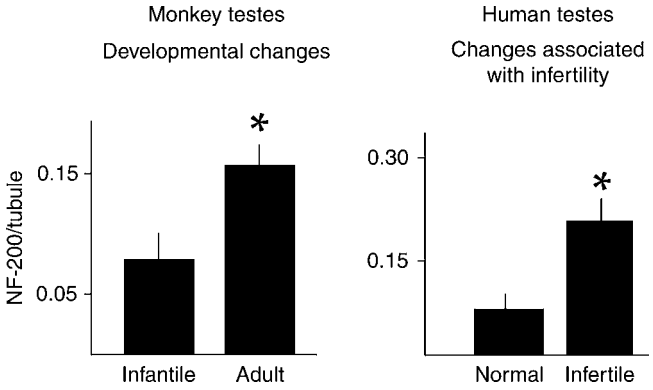


Fig. 2. Changes in density of testicular innervation. Graph depicting developmental changes in density of innervation (numbers of immunoreactive NF-200-positive nerve fibers per tubule) in rhesus monkey testes (left) and in testes of adult men suffering from defects of spermatogenesis and infertility (right). Asteriks denote statistically significant changes. For more data and details, *see* refs. *17,24*.

marking periods of specific action? And are neuronal signaling molecules causally involved in the development or maintenance of states of infertilty in human testes? Or, is it possible that on the contrary these changes may indicate compensatory mechanisms, for example, to counteract activation and actions of immune cells? In many inflammed tissues such a mechanism has been suggested to occur (*see* ref. *42* for details and references). Clearly, immune cells, namely mast cells and macrophages, which are also frequently increased in testes of infertile men (e.g., refs. *43,44*), and most likely bear receptors catecholamines and histamine, give circumstantial evidence for such an assumption. These and other intriguing possibilites will have to be addressed in the future.

Intratesticular Production of Neuronal Signaling Molecules

During the last decades several mainly immunohistochemical studies have suggested that cells of the testicular interstitium are producers of neuronal signaling molecules. For examples, it was proposed that Leydig cells of the human testis produce catecholamines (e.g., refs. *45–47*). This assumption is based on immunoreactivity for example, TH and DBH. However, these results have not yet been substantiated by other methods (e.g., by *in situ* hybridization and so on). Own work *(17,24,41)* also using immunohistochemistry in paraffin-embedded human and monkey testes, but also other studies in human testis *(23)*, failed to find TH-immunoreactive Leydig cells, whereas clearly showing this enzyme in nerve fibers (Fig. 1). Whether Leydig cells or a subgroup

of them not detected in these studies could be intratesticular producers of this neuronal signaling molecule remains to be fully elucidated. It should be mentioned that Leydig cells express a series of genes formerly thought to be "neuronal markers," including the neural cell adhesion molecule (NCAM; e.g., ref. *48*).

Experiments conducted in the rhesus monkey and in the human testis *(17,41)*, did not indicate Leydig cells as a source of catecholamines, but rather showed, occasionally, a phenotypically distinct cell type. It expressed TH and DBH and contained norepinephrine (shown by a histochemical procedure in monkeys). This cell type appeared elongated and may represent a neuron-like cell type. In human testes, these cells also are immunoreactive for the cell membrane-associated dopamine transporter and a voltage-activated sodium channel *(17)*. Interestingly, a similar, neuron-like cell type, expressing TH and other neuronal markers (including the receptor for nerve growth factor, NFs, and neuron-specific enolase) was reported in the ovary of primates and some strains of rats (*see* ref. *49*). Nevertheless, the precise nature of these cells in the male and female gonad remains to to be fully investigated.

GABA and Histamine

Evidence for local intratesticular production of neuronal signaling molecules is documented for GABA and histamine. In either case the situation is complicated by results indicating the presence of synthesizing enzymes in both tubular and interstitial compartments (*see* later; refs. *50–52*). Whether either signaling molecule may leak out to affect Leydig cells is unknown and tubular production will not be discussed here further. Regarding GABA, which is well documented in testis of rodents, changes of its levels occured with sexual maturation in hamsters (comparable to the situation for norepinephrine). Highest levels were found in maturing hamsters, not in adults *(53)*, a result that may argue against a sole contribution of tubular sources and possibly for a special role in development. Although a supply of GABA to the testis by innervating nerve fibers cannot be completeley ruled out (e.g., it might be contained in clear vesicles reported in testicular nerve fibers; e.g., ref. *15*), sources of GABA in the testis were recently identified as Leydig cells in the rat, mouse hamster, and human. A combination of cellular and molecular approaches unequivocally showed that Leydig cells express the synthesizing enzyme glutamic acid decarboxylase (GAD) with species differences being apparent with respect to GAD isoforms, GAD65, or GAD67. Furthermore, the vesicular GABA transporter (VGAT), necessary for packing GABA into storage vesicles, is present in Leydig cells. GAD is active, both in whole rodent testes during postnatal development and in adults, as well as in TM3 murine Leydig tumor cells *(53–56)*.

Histamine is used by neurons of certain brain ciruits as a neurotransmitter, but is also present in the testis. Older studies have indicated that the levels are the highest in immature animals *(37)*, suggesting a possible role at this period. Histamine can be produced by testicular mast cells, as shown recently by a laser microdissection study of the tryptase containg mast cells in human testes *(57)*. Besides this protease, which constitutes the major product of human mast cells and can thus be used to identify this cell type, these mast cells express the synthesizing enzyme histidine decarboxylase (HDC). Mast cells in the interstitium of the testis *(58,17,24)*, like many other tissues, are closely associated with nerve fibers and regarding function they are mutually interlinked. Thus neuronal signals may trigger release of mast cell products (*see* e.g., ref. *42*) and mast cell products can affect nerve fibers. Mast cells are normally rare in the interstitial spaces of the testis of the adult rat, but they become a common cell type after testicular trauma or treatment with ethylene dimethane sulphonate *(21)*. In comparison, these cells appear to be encountered much more often in the normal human testis *(43,58,59)* or rhesus monkey testis *(17,24)*. In the latter age-related changes became apparent. Increases were recorded especially with sexual development, which coincide with increased numbers of nerve fibers. Importantly, in the testes of patients diagnosed with idiopathic infertility, mast cells are reported to be dramatically increased in number over comparable normal men and and electron microscopy showed clear signs of activation, i.e., active secretion in states of infertility (*see* ref. *43*). Therefore, this signaling molecule (as well as other mast cell products; *see* ref. *59*) may be of as yet unexplored relevance in infertile testis.

Evidence for Regulation of Leydig Cells by Neuronal Signaling Molecules

Sources of neuronal signaling molecules in the interstitial compartment of the testis, i.e., close to Leydig cells, as well as delivery of adrenal-derived catecholamines through the blood flow, provide a solid basis for the notion that Leydig cell may be subject to their influences, provided that Leydig cells posses functional receptors (*see* ref. *1*). There are some newer pieces of information available in this respect, mainly from studies of gene expression in testes or in isolated Leydig cells *(60–62)*. These indicate, for instance, presence

and different levels of a nicotinic acetlycholine receptor subunit gene depending on age. Furthermore, in the case of GABA receptors, reverse transcription-polymerase chain reaction studies were performed *(63)*.

Catecholaminergic Influences on Leydig Cell Development and Function

When discussing the evidence for catecholaminergic receptors, it must be pointed out that it is mainly data from binding studies or pharmacological studies in vivo and in vitro and only from few molecular studies (e.g., ref. *64*). Furthermore, these represent a species mix (mouse, rat pig, few in human). It is accepted that Leydig cell are endowed with a β_2-adrenoreceptor *(65–72)* and possibly an α-adrenergic receptor *(40,73,74)*. An α-receptor is also expressed by myoid cells *(75)*, while Sertoli cells are endowed with the β_1-adrenoreceptor *(64,76)*, muscarinic ACh receptors *(77)* and possibly dopamine receptors *(78)*. Furthermore other interstitial testicular cells (e.g., mast cells, *32*) most likely have β-receptors. Studies adressing functionality of these receptors with respect to Leydig cells were summarized in detail in a previous review *(1)* and will be addressed in here only in brief.

Evidence From In Vivo Studies

Catecholamines may be important during development. This notion is suggested by high levels in immature testes (*see* "Plasticity During Development and Pathology") and indicated by consequences of surgical or chemical denervation (using the neurotoxin 6-hydroxydopamine), which all interfered with both normal testicular development and compensatory hypertrophy in immature animals *(79–82)*. Injections of newborn rats for 49 d with the β-adrenoreceptor agonist isoproterenol in contrast resulted in Leydig cell hypertrophy and increased testes weights *(83)*, implying a trophic role of catecholamines.

In adults, catecholamines may be important for regulation of Leydig cells function during stress, i.e., conditions associated with activation of the sympathetic nervous system. Reports are sketchy, but surgical denervation of the testis and adrenalectomy (which would reduce the blood catecholamine levels), interfered with the typically observed acute testosterone increase to acute stressful stimuli *(84)*, implicating a catecholaminergic component in acute stress-induced increases of androgen production. During longer lasting, chronic stress situations, by contrast, testicular function in adults is typically reported to be suppressed (*see* ref. *39*). This suppression can occur when LH-concentrations are normal *(85)* or even elevated *(86)*, hinting a contribution of catecholamines reaching the testis through blood flow and/or testicular innervation. For example, in support for the first mentioned possibility, a study on men suffering from head injuries (ref. *87*) revealed low androgen levels associated with high concentration of catecholamines in the circulation (*see also* ref. *39*). Yet, specific involvement of testicular innervation is suggested by results of heterogenous experimental approaches, including endocrine and compensatory responses to unilateral orchidectomy *(81,88)* and more recently by studies using intraventricular injection of interleukins or ethanol, which resulted in reduced steroidogenesis *(89,90)*. Furthermore, results of destruction of the postganglionic, secondary neuron innervating the rodent testis by chemical or surgical denervation of the testis, suggested that testicular innervation could regulate LH receptors, testosterone production as well as spermatogenesis and in vitro responsiveness to hCG and catecholamines *(39,91)*. Importantly spinal cord injury (around or higher the T12 region, where the preganglionic, first sympathetic neuronal cell bodies relevant for innervation of the testes are presumably located), is often associated with impaired spermatogenesis in rats and men *(92–94)*, a fact lending further support to the importance of testicular innervation, as part of the neural pathway between brain and testis, for testicular function in adult testis.

Besides Leydig cells, also other cell types including Sertoli cells may be affected by altered activity of testicular nerves or altered blood levels of catecholamines. Both blood vessel and nerve terminals present at the myoid cells and the basal lamina of tubules *(18)* provide an anatomical basis for such an assumption. Which testicular catecholaminergic receptor type(s) are responsible for these effects in vivo remains to be shown. That yet other effects of catecholamines (e.g., on testicular blood flow) must be considered is illustated by several in vivo studies. For example, injection of a β-adrenoreceptor agonist directly into the arterial supply of the testis increased testosterone in the dog *(95)*, whereas intravenous, subcutaneous or intra-arterial administration of epinephrine (acting both on the β- and the α-adrenoreceptor) resulted in decreased testosterone levels in men *(96)* and rats *(97)*. It is interesting that despite data implying testicular catecholamine receptors, the possibility of side effects, for example, widely used β-receptor antagonists on human testicular functions has not been addressed.

Cell Culture Studies

These studies have been performed in many species of different developmental phases (adult rats, mice refs. *65–67,98*; fetal mice refs. *70,71,99*; or immature pigs

ref. *72*), a fact, which may explain in part divergent results. Furthermore different experimental approaches have been chosen, for instance, purified Leydig cell cultures, dispersed interstitial cells or studies with testicular fragments (for details *see* ref. *1*). Most of these studies have found that catecholamines through action on the β-receptor increase production of the main male steroid, testosterone. However, freshly isolated Leydig cell cultures do not respond to catecholamines acting on β-receptors with increased production of androgens, but acquire responsiveness with time in culture *(68,100)*. Studies using dispersed testicular interstitial cells showed that epinephrine and the α-adrenoreceptor agonist phenylephrine did not affect testosterone of freshly cultured dispersed interstitial cells, but enhanced the action of hCG on testosterone production. Thus, α-receptors present on unidentified cells are also involved. When testicular tissue fragments were studied in hamsters *(39,40,101–103)*, the natural catecholamines epinephrine and norepinephrine, and α-adrenoreceptor agonist, phenylephrine, augmented the stimulatory effect of hCG, while the β-adrenoreceptor agonist isoproterenol did not. In line with these data, elevations of cAMP (i.e., the second messenger of β-receptors) were not involved in the stimulation of testosterone by catecholamines. Thus, besides involvement of β-receptors, α-receptors mediate effects of catecholamines presumably through paracrine interactions between Leydig cells and other testicular cells. The most recent article studying purified hamster Leydig cells, interestingly reports that based on the specificity of commonly used drugs, both α_1- and β_1-adrenoreceptors, rather than the presumed β_2 receptors in other species are involved in the catecholamine mediated rise of testosterone *(4)*.

The functional or developmental state of the testis is of importance, because both α_1- and β-receptor-mediated effects on testosterone production were found in the prepubertal (17-d-old) testes of golden hamsters *(40)*, results that are similiar to those reported for the rat *(73,74)*. Furthermore, in the 17-d-old golden hamster testes, as in regressed testes of short-photoperiod (SP)-exposed hamsters (a model for physiological infertility) and in testes of both long photoperiod (LP-) and SP-exposed Siberian hamsters, catecholamines did not only synergize with hCG to further increase its effect on testosterone production, but were active alone. Concentrations of catecholamines, as low as 10 nM were effective, i.e., levels matching the concentrations of norepinephrine in golden hamster testicular homogenates. Indeed much higher concentrations can be expected locally in the interstitial compartment of the testis, thus, the effects of catecholamines observed in these studies are likely to be of physiological significance.

Genetic Approaches

Currently, to my knowledge, modern approaches to study testicular catecholaminergic receptor subtypes are still missing, possibly because of the lack of Leydig cell specific knockout models. Whole "knockout" approaches both for receptors or catecholamine synthesizing enzymes are normally not suitable for meaningful insights, because of massive and body-wide consequences (*see* for instance results of catecholamine synthesizing enzyme or receptor knockouts, e.g., refs. *104,105*).

Hints to involvement of catecholamines in testis development and Leydig cell function come from a naturally occuring mutant, the BALB/cJ mouse, with high levels of catecholamine-synthesizing enzymes (*see* ref. *106*). Adrenal norepinephrine concentrations in BALB/cJ mice are significantly elevated and testes are significantly larger, in comparison with a normal strain of closely related BALB/cByJ mice. Overall testosterone levels were normal, but conversion of 17-OH-progesterone to testosterone was lower, suggesting inhibition of this step by norepinephrine, possibly as an adaptation to high catecholamine levels *(106)*.

Summary

Although the reports available are at first glance providing a rather heterogeneous picture of the role of catecholamines, both in vivo and in vitro results support a direct effect of catecholamines at the testicular and Leydig cell level. Yet nature and degree of this influence change. During development, high levels of catecholamines and evidence for their actions together imply that catecholamines act as trophic factors in the testis (*see* ref. *107*). In regard of steroidogenesis, the differences between (functionally) immature and (functionally) mature testes indicate that catecholaminergic influences are important in periods when the gonadotrophic stimulation is missing or reduced. Catecholamines may thus provide both a stimulatory input for Leydig cell steroidogenesis during development and a "back-up" system in SP animals. In addition, and in line with the view of a backup system, the catecholaminergic system may enhance the stimulatory effect of hCG/LH on steroid production in the adult and thus, may have a stimulatory role in acute stress. Far less is known about chronic stress, but catecholamines may also be testicular mediators in such phases, in which by unknown mechanism

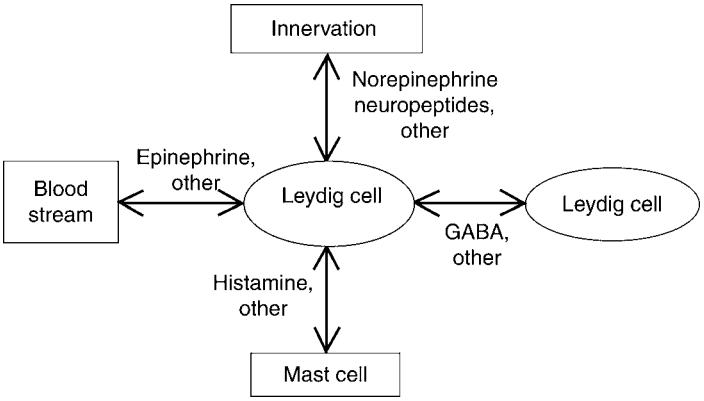


Fig. 3. Sources of the main neuronal signaling molecules able to affect Leydig cells and interactions. Direct or indirect innervation supplies catecholamines and neuropeptides and delivers them close to Leydig cells. Leydig cell–Leydig cell interactions in paracrine and possible autocrine fashions, for example, through GABA can be assumed. Other interstitial cells, such as mast cells, are interacting with nerve fibers. Mast cells for example, release histamine, which then can act on Leydig cells. Furthermore, catecholamines and a plethora of other signaling factors are blood-born and may affect all interstitial cells. Further mutual interactions can be presumed, but like developmental changes, they are not indicated for reasons of clarity.

suppression of Leydig cells function may result. Several subtypes of catecholaminergic receptors are present in the testis on different cells, maybe even together on (some?) Leydig cells. The situation is even more complex, because multiple interactions between, for example, immune cells, nerve fibers, and Leydig cells are likely to occur in the testis, as in other tissues, and must also be taken into consideration (*see* Fig. 3). Thus depending on the develpomental or functional state, catecholamines may be trophic for Leydig cells, or may be positive or in contrast negative regulators of steroidogenesis.

Neuropeptides (VIP, NPY, and CGRP) and ACh

VIP and NPY (Fig. 1) can be colocalized in noradrenergic fibers in the testis, although only very few of these immunoreactive fibers were reported *(2,17,19, 22,24,108)*. In neonatal rat testicular cultures, VIP stimulates androgen biosynthesis *(109)*, but may be more important as endocrine regulatory factor of fetal rat testicular steroidogenesis (*see* ref. *110*). Similiar studies in the adult are not known, but based on the typical roles of VIP, it may be involved in regulating smooth muscle function and thus blood flow. A structurally closely related peptide, pituitary adenylate cyclase-activating polypeptide, is expressed at high levels in germ cells, where it locally activates cAMP-coupled receptors of Sertoli cells *(111)*. Whether it may leak out of the tubular compartment and affect Leydig cells is not known. The author is not aware of published studies addressing the roles of NPY, described in many studies to be present in testicular nerve fibers, on Leydig cell function. It should be mentioned that while NPY mRNA was not found in freshly isolated immature Leydig cells, NPY mRNA levels were detected after the addition of LH or cytokines *(112)*. CGRP may mark sensory, afferent fibers of the testes, as mentioned. Calcitonin receptor-like receptor, which was recently found to mediate the effects of both CGRP and adrenomedullin, is reported to be expressed in human Leydig cells *(113)*. In view of the ligand being present in presumably few sensory fibers of the testis, it is not clear in which way it might affect Leydig cells. Instead Lissbrand et al. *(26)* clearly showed that CGRP is a potent vasodilator in the testicular vasculature affecting extratesticular blood vessels. ACh is the prototype neurotransmitter of the postganglionic parasympathetic neuron and despite weak evidence for parasympathetic innervation of the testis (*see* summary in ref. *2*), there is some evidence for action on isolated Leydig cells. Thus basal and hCG-induced testosterone release by Percoll-purified Leydig cells of the rat were decreased by ACh and its analog carbachol (acting on muscarinic and nicotinic ACh receptors), as well as by nicotine *(114)*, which activates only the nicotinic subfamily of ACh receptors. This and another study *(115)* indicated that nicotinic, but not muscarinic agonists inhibit androgen biosynthesis through selective inhibition of 17α-hydroxylase activity. Interestingly, the gene for the nicotinic α_4-subunit receptor was recently identified to be among upregulated genes associated with Leydig cell development *(60)* and to be downregulated with aging *(61)*. It remains to be shown whether indeed cholinergic innervation exist *(16)* or whether local intratesticular production of ACh occurs. The existence for the latter possibility is suggested for the tubular compartment

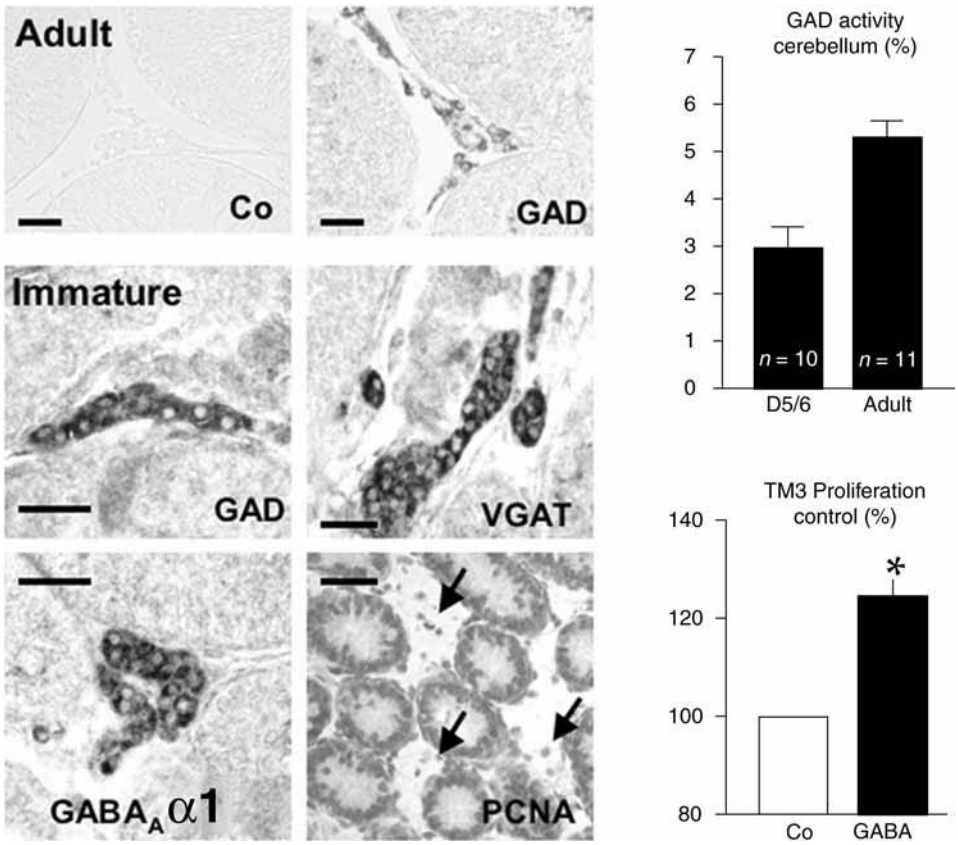


Fig. 4. The GABAergic system of the testis. The GABA synthesizing enzyme GAD is present in interstitial cells of the adult rat (top two micrographs; Co: negative control) and in the immature rat gonad (Day 5; middle left panel). Interstitial Leydig cells also contain the intracellular vesicular GABA storage transporter (VGAT; middle right micrograph). GAD is active both in adult and immature testes, as shown by an enzymatic assay (top graph; data expressed as percent of activity in brain). Interstitial cells posses $GABA_A$ receptor subunit-α_1 (bottom left) and in the immature testis, interstitial cells also proliferate (arrows in bottom righ micrograph showing the proliferation marker PCNA). GABA appears to be linked to Leydig cells proliferation (bottom graph) and in TM3 cells GABA statistically significanty stimulates proliferation, as measured using a proliferation assay. Bars approx 50 µm. For further details, *see* refs. *54, 55*.

(116) and clearly occurs in the ovary, namely in the granulosa cells of the ovarian follicle *(117)*.

GABA

GABA, mainly known as the predominant neurotransmitter in the brain, has emerged during the last decades as a local signaling molecule of endocrine organs (*see* refs. *54,55*). Production sites of GABA are usually close to its targets, bearing the different receptor types (especially $GABA_A$ and $GABA_C$, which are ion channels, and $GABA_B$, a metabotropic receptor). GABA and its receptors subtypes or subunits are present in the rat and hamster testes and Leydig cells *(63,118,119)*, thus implying a local intratesticular GABAergic system. Some progress has been made in the elucidation of the role and composition of this system, which appear to depend on the functional stage of Leydig cells. Studies in adult Leydig cells showed that stimulation of testicular steroidogenesis in decapsulated testes in vitro is a consequence of GABA action and that GABA acts on interstitial cells, through $GABA_A$ and $GABA_B$ types *(118,119)*. Work from our group has readdressed this topic (Fig. 4). Based on the fact that GABA levels are highest in developing testes *(53)* and that a full GABAergic system (i.e., GABA production, storage prerequisites and $GABA_A$ receptors) is present at this time in interstitial cells of rodents *(54,55)*, we examined effects of GABA and $GABA_A$ agonists were on Leydig cells proliferation. At least in TM3 Leydig cells, we found clear evidence for a stimulatory role of GABA and a $GABA_A$ agonist on cell proliferation. This cell line may be comparable with the proliferating Leydig cell precursor cells of the postnatal testis (*see* ref. *120*), which coexpress a proliferation marker (proliferating cell nuclear antigen [PCNA]), the GABA biosynthesis enzyme, GAD, and GABA receptors. These

results have led to the hypothesis that locally produced GABA may be involved in the development of the adult type Leydig cell population (*see* refs. *54,55*). Ongoing work is attempting to identify the cellular mechanisms of $GABA_A$ mediated action of GABA in Leydig cells. It appears that the induction of transcription factors (Doepner and Mayerhofer, unpublished; and *121*) are part of the GABA action. Interestingly, one GABA-induced factor (early growth response factor-1) is present in proliferating precursor Leydig cells and was recently independendly identified to be downregulated when Leydig cells develop *(60,121)*. Thus like in other developing organs, including brain, GABA may act as a tropic molecule involved in cell growth and differentiation of Leydig cells. Many aspects of the GABA-system in Leydig cells are currently unknown. For example, it remains to be tested whether steroids may bind and signal through GABA receptors of Leydig cells *(122)*. However, it should be mentioned, that a GABAergic system is also present in normal as well as infertile human testes *(54)*, a result that calls for a thorough examination of the testicular role of GABA. In view of the fact that many clinically used drugs can interfere with GABA signaling and action (*see* ref. *54*) and that steroidogenesis may also be affected, this point deserves attention.

Histamine

Only few studies have so far been interested in the possibility that histamine may affect Leydig cell function and development. An interesting observation is that general HDC knockout mice have altered intratesticular steroid levels *(123)*. That and in part how histamine can increase and/or decrease Leydig cells testosterone production was shown in two species. Stimulatory effects on the production of testosterone and progesterone (a testosterone precursor) by hamster testicular fragments in vitro became evident using short time testicular incubations *(124)*. Histamine enhanced the hCG-induced stimulation of steroid production. Histamine alone stimulated steroidogenesis in a dose-dependent fashion in the regressed, i.e., functionally immature testes of SP-exposed animals, but not in the functionally active testes of LP-exposed hamsters. These histamine effects appeared to be mediated through a H1-receptor, indicated by pharmacological blockage with a H1R-antagonist. Deeper insights into direct effects of histamine of Leydig cells come from a recent study. Mondillo et al. *(125)* studied purified rat Leydig cells and report both H1- and H2-mediated effects in the regulation of Leydig cell steroidogenesis. These receptors are activated in a dose-dependent manner and their activation produces stimulation or inhibition of steroidogenesis. From these few available reports it is already becoming clear that within the testis, histamine effects are either direct on Leydig cells and/or indirect because of the intermediary and paracrine action of another cell type acting on Leydig cells. Indeed, H1- and H2-receptors are found in cells of all major testicular compartments, interstitial, tubular and peritubular, at least in human testis *(57)* as evidenced by a recent laser microdissection/reverse transcriptase-polymerase chain reaction study. This novel fact calls for a thorough investigation of effects of widely used antihistaminic drugs, which based on these new results, might in theory be able to interfere with testicular function. Importantly, in the rat such a "side effect" of a particular H2-receptor antagonist (which is a widely used drug in human), was recently shown and resulted in massive derangements of spermatogenesis *(126)*.

CONCLUSIONS

The roles of neuronal signaling molecules in the mammalian testis are far from being understood, but because previous reviews on this topic appeared *(1,127)*, progress has been steadily made, for example, in respect of the neural connection of the brain to the testis and regarding further identification of the nature of some of the signaling molecules present in testicular nerves, neuron-like cells, and in nonneuronal testicular cells. It is becoming increasingly evident that innervation and nonneuronal sources of signaling molecules, as well as specific actions of these factors, change during development. Furthermore, changes occur in testes of men with impaired spermatogenesis and it has been speculated that they may be involved in the pathogenic processes underlying infertility in men. Furthermore, the identification of intratesticular signaling systems involving catecholamines, GABA, histamine, and their receptors in men may change and expand the perception of this topic. Thus, this area emerges as one bearing potentially clinical relevance. Deeper insight may allow to judge whether clinically used drugs, which interfere with the GABA-, histamine-, or catecholaminergic systems, may be able to directly or indirectly influence the function of the androgen-producing Leydig cell, and consequently spermatogenesis and male reproduction.

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21 Regulation of Leydig Cell Function as it Pertains to the Inflammatory Response

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SUMMARY

The immune–endocrine interactions that govern Leydig cell function are described in relation to both positive, trophic interactions of immune regulatory effects during development and in the normal, healthy noninflamed testis, and inhibitory effects of the same mediators in response to inflammation, infection, and chronic illness. The unique immune environment of the testes is detailed with particular emphasis on the functional significance of the close physical association between testicular interstitial macrophages and Leydig cells. The role of four classes of inflammatory mediators in the regulation of Leydig cell functions is discussed in detail: proinflammatory cytokines, reactive oxygen species, nitric oxide, and prostaglandins. The role of immune-dysregulation in the decline of steroidogenic function is addressed in the context of how macrophage–Leydig cell interactions change in the aging male.

Key Words: Aging; cyclooxygenase-2; inflammation; macrophages; reactive oxygen species; steroidogenesis.

INTRODUCTION

Leydig cells and macrophages in the interstitial tissue of the testis were first observed in intimate association in the late 1960s. This close physical association suggests that testicular interstitial macrophages and Leydig cells are functionally related. Over the past decade numerous investigations have examined the physiological and functional significance of macrophage–Leydig cell associations, yet much remains to be learned. Several important concepts have emerged. The unique, intimate physical association of testicular interstitial macrophages with Leydig cells as yet defies explanation. It is clear that the immune environment within the testis is highly specialized. The seminiferous tubules form the blood–testes barrier, which physically excludes immune cells from contacting allogenic spermatocytes during development. But the testicular interstitium, where Leydig cells reside, is freely accessible to the peripheral circulation and is freely permeable to not only resident macrophages, but other leukocytes including circulating monocytes, lymphocytes, neutrophils, and natural-killer cells. Despite this access, the testicular interstitium is an immunologically privileged site, as is the seminiferous epithelium. There is a paradox of immune–endocrine interactions in the testis—it is an immunologically suppressed environment yet it is capable of a robust inflammatory response. Testicular macrophages produce factors, which have diametric effects on Leydig cells, both promoting developmental changes and differentiation of immature cells, whereas inhibiting differentiated functions of adult cells. In addition to inflammatory mediators which inhibit Leydig cells functions, macrophages, also, produce additional factors under noninflammatory conditions, which promote adult Leydig cell functions. This chapter will provide an update on Leydig cell–macrophage interactions *(1–3)*, discuss the dual role that inflammatory mediators play in the regulation of adult vs immature Leydig cell functions, and will examine the importance of immune dysregulation as a contributing factor to age-related decline in Leydig cell steroidogenesis.

IMMUNE–ENDOCRINE INTERACTIONS IN THE TESTIS

The immune system within the testis appears to be normal with effective lymphatic drainage and relatively

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free access of immune cells to the interstitial tissue *(4,5)*. There also exists a very effective blood–testis barrier located within the meiotic cell compartment *(6,7)*. These physical constraints do not appear to account for the immunological protection in the testes across a range of species. Studies over a number of years have shown that the testis constitutes a unique immunoregulatory environment, which exist to provide protection for the endogenous antigens of spermatogenesis; protection which also extends to foreign antigens on grafts inserted into the testicular environment *(8,9)*. Immune cells which find their way into the testis appear to be functionally modified to restrict their proinflammatory activity and provide an immunologically suppressed environment where immune responses are blunted and/or limited.

The intimate interaction between the male reproductive tract and the immune system contributes to reproductive dysfunction, which is not only associated with local infection and its accompanying inflammation but also with systemic disease and inflammation *(10,11)*. Consequently, men with a wide range of systemic illnesses generally display reduction in both serum androgen levels and sperm output. This indicates that male sexual function and general well-being operate in a reciprocal relationship, which may even represent a physiologically important regulatory mechanism. The yin–yang relationship between the male reproductive endocrine system and immune system has been described in terms of "sickness behavior," when the immune system is dominant and "testosterone behavior" under normal reproductive conditions, when libido and feelings of well being dominate *(2)*.

Macrophages are commonly observed in the interstitium of most, if not all species, and many testes also contain variable numbers of mast cells and/or eosinophils *(12,13)*. Less numerous, but ubiquitous nonetheless, are the intratesticular lymphocytes *(14,15)*. The presence of these cells clearly demonstrates that immune cells have unopposed access to the testicular parenchyma. The resident testicular macrophages have been extensively studied, initially by Miller and colleagues *(16,17)* and subsequently by, Hutson and colleagues *(18,19)*. These cells were found to share all the classical characteristics of macrophages in other sites, but to possess testis-specific features as well. Testicular macrophages have been most extensively studied in the rat, and to the lesser extent in the mouse, with relatively few investigations in other species. Other species with large numbers of testicular macrophages include the guinea pig, hamster, horse, bull, and human *(20,21)*.

Interest in macrophage–Leydig cell interactions was initiated when morphological examination of the adult rat testis first indicated that there was a direct structural interaction between these cells *(22)*. Cytoplasmic processes of Leydig cells were observed, which extended to membrane invaginations of adjacent macrophages *(13,22,23)*. These specialized membrane associations have been termed "digitations" *(13)*. In the rat and mouse, the ratio of macrophages to Leydig cells appears to be relatively fixed at approx one macrophage to every four or five Leydig cells *(24,25)* and macrophages display a very close physical and functional relationship with the Leydig cell clusters. Ultrastructural studies have established the existence of highly-specialized cytoplasmic interdigitations linking the two cell types, indicating the potential for direct exchange of information and material *(18,26–28)*, whereas macrophages and Leydig cells undergo parallel alterations in morphology and cytoplasmic volume in experimental models of cryptorchidism and vasectomy in the adult rat *(29,30)*. Figure 1 demonstrates the close physical association of Leydig cells and macrophages in the rat testicular interstitium. It has been suggested that testicular macrophages metabolize Leydig cell secreted steroids *(23)*, as has been shown for peritoneal macrophages, which can convert androstenedione to testosterone and DHT *(31)*.

Testicular resident macrophages possess all the structural and functional characteristics of macrophages found in other connective tissues *(13)*. They display the characteristic nuclear and cytoplasmic morphology of the mononuclear phagocyte lineage, are actively phagocytic, bactericidal and adherent in culture, and they express many macrophage-specific enzymes, cytokine receptors, and surface antigen markers *(17,25,28,32–35)*. Many studies suggest that there is a testis-specific phenotype for these macrophages. In addition to presumptive roles in connective tissue remodeling, phagocytosis of cellular debris and maintenance of innate immunity in the testis, studies by several laboratories suggest that these cells play an important role in local immunoregulation *(36,37)*. Importantly, macrophages play an essential role in normal Leydig cell development and in maintaining Leydig cell steroidogenic function in the adult *(38–40)*. The immunological functions of the testicular macrophage population have been investigated. Production of several key inflammatory mediators, including interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, inducible or macrophage-type nitric oxide synthase (iNOS), and macrophage migration inhibitory factor (MIF), by rat testicular macrophages in response to stimulation by Gram-negative bacterial

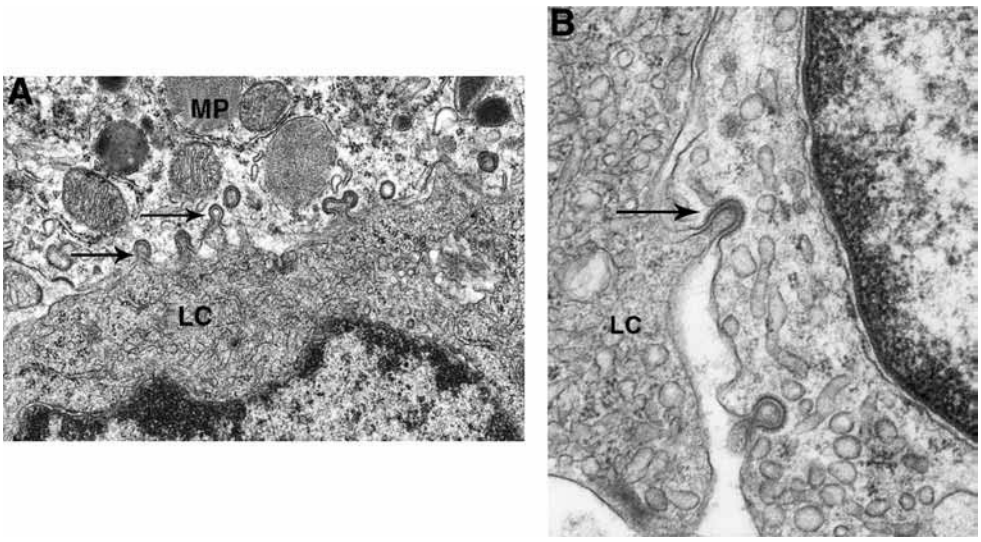


Fig. 1. Intimate physical association of adult Leydig cell (LC) and resident testicular macrophage showing processes extending from LC onto macrophage (MP). **(A)** Process extending from LC is frequently seen inserted into coated membrane invaginations (arrows) on the MP surface. ×33,800 **(B)** More detail of LC processes inserted into coated membrane invagination (arrow) in the MP. ×96,300 (Reprinted with permission from ref. *23*).

endotoxin, lipopolysaccharide (LPS), is considerably blunted, relative to peritoneal macrophages *(36,41,42)*. In contrast, studies on inflammation in the mouse testis have demonstrated that these macrophages express IL-1β mRNA and produce bioactive TNF-α at fairly significant levels upon LPS stimulation *(43,44)*. Studies have documented the appearance of several populations of macrophages in the rat testis, which may represent different stages of development and/or functional states *(37)*. Studies on rat macrophages have utilized specific antibodies able to distinguish between inflammatory-infiltrating monocyte/macrophages (ED1) and resident tissue macrophages (ED2). Compared with the $ED1^-$ macrophages, the $ED1^+$ subset possesses a distinctly smaller nuclear diameter and displays significant levels of expression of iNOS in both normal and LPS-stimulated rats *(45)*. Similarly, expression of IL-1β protein after LPS-treatment appears to be confined to a relatively small subset of rat testicular macrophages *(42)*. The testis contains subsets of macrophages with varying proinflammatory capacities. However, most investigations on testicular macrophages to date have used mixed populations in order to study their function. The lack of specific antibodies able to distinguish subsets of mouse macrophages has hampered similar investigations in the mouse.

Inflammation and activation of the immune response has immediate and mostly negative effects on male reproduction function *(46–52)*. The effects of inflammation on male reproduction can be separated largely into effects on testicular steroidogenesis leading to androgen deficiency problems and effects on spermatogenesis resulting in reduced sperm output. Inflammatory mediators, which have been shown to affect Leydig cell function include the cytokines IL-1, IL-6, TNF-α, and transforming growth factor (TGF)-β family members, small molecular weight mediators such as nitric oxide (NO), prostaglandins (PG) of the E series (PGE) and F series (PGF) *(53,54)*, and reactive oxygen species (ROS) *(55–58)* Cytokines in particular have been implicated as growth and differentiation factors in both compartments of the testis *(3,59)*. They also play a role in modifying the endocrine control of the testis, through actions at multiple levels within the hypothalamic–pituitary–testis (HPT) axis; for example, IL-1β inhibits steroidogenesis by suppressing hypothalamic GnRH production and Leydig cell steroidogenesis directly *(60,61)*.

THE ROLE OF MACROPHAGES IN LEYDIG CELL DEVELOPMENT

There seems little doubt that macrophages play an important role in Leydig cell development. There is a close temporal link between the maturation of the adult Leydig cell population and the increase in the number of testicular resident macrophages during puberty

(24,62,63). Moreover, specific depletion of the intratesticular macrophages inhibits the development of Leydig cells in immature rats, and the recovery of Leydig cells after EDS-treatment in adult rats *(39,64–66)*. The *op/op* mouse, which has an inactivating mutation in the M-CSF gene and consequently has reduced numbers of macrophages throughout the body, including the testis, also displays very poor fertility and reduced testicular testosterone production because of developmental and steroidogenic abnormalities of the Leydig cells *(67,68)*.

The role of the testicular macrophages in Leydig cell growth and differentiation may involve the intercytoplasmic specializations between the two cell types, which appear very early in adult testicular development in the rat *(18)*, and/or specific macrophage-derived cytokines. In cultures of isolated Leydig cells from rats 10–20 d old, IL-1β caused a dose-dependent increase in DNA synthesis as measured by incorporation of *(3)* [H]-thymidine *(69)*. IL-1α also had an effect, although, it was much less potent than IL-1β. The effect of IL-1β was not observed in Leydig cells isolated from older animals, suggesting that macrophage IL-1β may play a role in the proliferation of Leydig cells during prepubertal development *(69)*. Collectively, the published data indicate that resting testicular macrophages have a positive or trophic effect on Leydig cell steroidogenesis.

Depletion of testicular macrophages results in impaired Leydig cell development and differentiation, indicating that macrophages secrete factors, which promote Leydig cell functions. In addition to IL-1, other macrophage secreted factors are likely involved. IGF-1-null mice have diminished numbers of Leydig cells and lower circulating testosterone levels in adult males *(70)*. Macrophages are known to spontaneously produce and secrete IGF-1, which contributes to wound healing and tissue remodeling *(71)*. These observations are consistent with a role for macrophage-secreted IGF-1 in promoting Leydig cell differentiation and proliferation.

Testicular macrophages have been shown to express the cholesterol 25-hydroxylase enzyme and produce 25-hydroxycholesterol *(72,73)*. It has been proposed that testicular macrophages, because of their close association with the Leydig cells, provide 25-hydroxycholesterol as a substrate for testosterone biosynthesis, bypassing the need for steroidogenic acute regulatory protein (StAR) and supporting basal steroidogenesis. Interestingly, the 25-hydroxylase enzyme is negatively regulated by testosterone suggesting that there may be a feedback loop between the macrophages and Leydig cells *(74,75)*. Moreover, it has been suggested that one of the factors, which macrophages secrete in support of Leydig cell proliferation and development may be 25-hydroxycholesterol *(76)*. It is possible that as Leydig cells age and the digitations with macrophages are lost (*see* p. 313), the ability of macrophages to deliver hydroxycholesterol to the Leydig cells is diminished, and this may contribute to steroidogenic senescence that accompanies aging.

INFLAMMATORY MEDIATORS INHIBIT ADULT LEYDIG CELL STEROIDOGENESIS

One of the most distinctive features of Leydig cells is their close physical association with interstitial macrophages. Macrophages are sentinels of the immune system and convey both positive and negative regulatory signals to Leydig cells. During inflammation, macrophages produce and secrete proinflammatory cytokines, ROS, NO, and PGs. All of these inflammatory mediators have been shown to inhibit Leydig cell steroidogenesis. Other products of the immune system, such as lymphocyte secreted cytokines are influenced by the inflammatory milieu of the interstitium and would be expected to contribute to the immune-suppression of steroidogenesis. The physical association between macrophages and Leydig cells may provide a conduit for transfer of macrophage products to the Leydig cell—and vice versa. As the testes age, these close physical associations or digitations are lost, presumably interrupting the direct exchange of macromolecules between these cells *(77)*. Whereas the transfer of inflammatory mediators is likely unimpeded by this disruption, the transfer of positive acting regulatory factors maybe preferentially blocked. This could result in favoring an increasingly more proinflammatory state in the Leydig cell, further contributing to the age-related senescence of steroidogenesis.

Proinflammatory Cytokines and Leydig Cell Function

Interleukin-1

The proinflammatory cytokine, IL-1 exists as two isoforms (IL-1α and IL-1β) with quite different structures able to bind to the same receptor to exert an almost identical range of effects *(78,79)*. Both isoforms induce the expression of numerous proinflammatory proteins, such as iNOS, cyclooxygenase (COX)-2, IL-6, and TNF-α *(78)*, and stimulate their own production as part of an amplification loop *(78,79)*. IL-1 is produced by many cells types, but activated monocytes and macrophages are the major source of secreted IL-1 in the body *(78)* Both IL-1α and IL-1β are single chain proteins, with only about 25% homology at the mature

protein level. They are synthesized as 31–33 kDa precursor proteins that are cleaved enzymatically to active 17 kDa forms. Both the precursor and mature forms of IL-1α are biologically active, but the IL-1β precursor is functionally inactive *(80–82)*. Conversion of IL-1β to the mature protein involves the action of a specific protease, called IL-1 converting enzyme or caspase-1, and processing is linked to its secretion into the extracellular space *(83)*. In contrast to IL-1β, the majority of IL-1α tends to remain within the cell or associated with the cell membrane *(78)*. The actions of IL-1 are mediated by the IL-1 type-I receptor (IL-1RI), a member of the Toll-like receptor superfamily *(84)*. In addition, there is an IL-1 type-II receptor, which is not linked to signal transduction but acts as a decoy receptor *(85)*. There is a third member of the IL-1 cytokine group that is homologous with IL-1α and IL-1β and binds to the IL-1 receptors, but lacks the ability to transduce a signal. This cytokine is called IL-1 receptor antagonist (IL-1ra), and appears to act as a competitive antagonist of IL-1 action *(86,87)*. Within the interstitial tissue, IL-1 is a regulator of androgen production *(88–90)*. All forms of IL-1α stimulate testosterone production by immature Leydig cells suggesting that Sertoli cell-derived IL-1α may be an important paracrine regulator of Leydig cells during testicular development. In contrast, locally injected IL-1β, but not IL-1α, induces acute inflammatory-like changes in the testicular microcirculation of adult rats *(91)*. IL-1 inhibits LH/human chorionic gonadotropin (hCG) and/or cAMP stimulated testosterone production *(2,44,90,92–97)*. In the mouse, the major site of inhibition occurs at the level of the P450c17 enzyme. In the rat, IL-1 appears to primarily affect Leydig cell steroidogenesis at the level of P450ssc, whereas StAR gene expression and protein synthesis are unaffected *(98,99)*. The effects of IL-1 on the Leydig cell may involve, in part, regulation of COX-2 enzyme expression and PGE_2 acting as an intermediate *(93,100,101)*.

In contrast to the inhibitory effects that IL-1 exerts on adult Leydig cells, this proinflammatory cytokine, in particular IL-1α, promotes Leydig cell proliferation, as described earlier, and is reported to stimulate steroidogenesis in immature Leydig cells. Investigations have examined the cooperation between IL-1α and GH and IGFs with regard to stimulation of steroidogenesis by Leydig cells from 40-d-old rats in vitro. Findings suggest that the stimulation of steroidogenesis in Leydig cells evoked by GH and IGFs requires cooperation with IL-1α. This cooperation may play an important role in connection with postnatal Leydig cell maturation and steroidogenesis *(102)*. Testicular IL-1α is expressed during development, primarily in Sertoli cells, appearing in rats for the first time 20 d after birth. This cytokine is microheterogeneous, consisting of three molecular species with molecular weights of 45, 24, and 17 kDa. The 17 kDa form represents mature IL-1α, whereas the 24-kDa IL-1α has been shown to be an alternately spliced form of the 45-kDa pro-IL-1α *(102)*. These isoforms of IL-1α were seen to stimulate basal testosterone production in immature Leydig cells, but not in the corresponding adult cells. This effect involved induction of the steroidogenic acute regulatory (StAR) protein and positively regulation by p38 MAPK. The findings suggest that isoforms of IL-1α may serve as paracrine mediators, alone or in concert with other factors which support proper testicular cell functioning *(103)*.

Inhibition of testosterone production is an important mechanism mediating inflammatory disease-associated decreases in male fertility. A lack of androgens will ultimately result in failure of reproductive function, not only because spermatogenesis is dependent on androgens for support, but also because maturation, transport, and ejaculation of spermatozoa rely on the activity of the androgen-dependent accessory organs and tissues. Men with critical illness, burn trauma, sepsis, and rheumatoid arthritis have elevated serum TNF-α and IL-1 levels and reduced testosterone levels *(104,105)*. Similar decreases in gonadal function have been reproduced in experimental animal models of chronic inflammation and systemic immune activation. Experimental adjuvant-induced arthritis *(106,107)* results in a dramatic reduction in serum testosterone levels in rodents and conditioned medium from testicular macrophages isolated from adjuvant-induced arthritic rats is inhibitory to Leydig cell testosterone production in vitro *(108)*. Intraperitoneal or intravenous injection of LPS in rats, mice, and rams or induction of sepsis with cecal slurry in male rats is also typified by a significant decrease in intratesticular and/or serum testosterone levels *(51,57,109,110)*. In rats, the early inhibition of testosterone is because of direct inhibition of Leydig cell steroidogenic function by LPS or its intermediates, whereas the later inhibitory phase may involve extratesticular effects, such as increased circulating inflammatory mediators or the release of endogenous glucocorticoids *(109,111)*. Similar inhibitory effects on serum testosterone levels can be induced in male rats by infusion of TNF-α alone *(112)*. In order to directly assess the effects of TNF-α on the HPT axis in human, van der Poll and colleagues *(113)*, injected six healthy men with recombinant TNF-α and measured serum concentrations of gonadotropins, testosterone,

and sex hormone-binding globulin. The results suggest that TNF-α affects the HPT axis at multiple levels in men and may be involved either directly or indirectly in the decrease in circulating testosterone concentrations in systemic illnesses *(113)*. In another study, male patients treated with high doses of IL-2 as therapy for metastatic cancer were found to have significantly reduced serum testosterone levels *(114)*. IL-2 causes the elevation of numerous serum cytokines, in particular TNF-α, IL-1, and interferon (IFN)-γ *(115,116)*. Finally, TNF-α levels are elevated in obese men and men with type-II diabetes *(117)*, conditions known to be associated with hypogonadism and decreased circulating testosterone levels.

Tumor Necrosis Factor-α

TNF-α is a 17 kDa glycosylated polypeptide secreted principally, by activated monocytes and macrophages *(118)*. It binds as a trimer to either of the two TNF receptors (TNFR1 and TNFR2), which are found on most cells in the body, and plays a central role in the initiation of the inflammatory response *(119)*. TNF-α stimulates the release of IL-1 and IL-6 from activated monocytes and macrophages and its synthesis and release is enhanced by IFN-γ secreted from activated T-lymphocytes *(120)*. There are two families of TNF-α receptors. The type-1 TNFRs induce cell death through a motif in their cytoplasmic regions called the death domain *(121)*. The TNFR type-2 receptors do not contain a death domain in their intracellular domains *(121,122)*. Treatment of isolated rat or mouse testicular macrophages with LPS-induced TNFα secretion *(43,123)*, indicating that TNF-α is produced by testicular macrophages under inflammatory conditions. There are a number of reports in the literature describing the effects of TNF-α on Leydig cell steroidogenesis *(89,112,114,124–126)*. Although one group concluded that TNF-α stimulates steroidogenesis *(124)*, the majority of these reports describe inhibitory effects and a decrease in the production of testosterone. These studies have been performed in a variety of systems, including intact animals *(112,114)* isolated primary cultures of Leydig cells *(126,127)*, and in MA-10 tumor Leydig cells transfected with Cyp17 (the P450c17 gene)-reporter constructs *(128)*. Inhibition of hCG binding by TNF-α has been reported *(129)* but the majority of these studies suggest that TNF-α inhibition occurs downstream of cAMP production at the level of steroidogenic gene expression. Results from several independent studies have also shown that the TNF-α inhibitory affects on Leydig cell testosterone secretion may involve a sphingomyelin/ceramide-dependent pathway *(130,131)*. More recently, the inhibitory action of TNF-α on Leydig cell steroidogenesis was shown to occur through the nuclear factor (NF)-κB pathway *(132)*, possibly in response to activation through protein kinase C *(128)*. The transactivation of the P450c17/Cyp17 gene by SF-1 and NUR77 was inhibited by NF-κB activation, suggesting that perturbation of these transcription factors is the most distal event in TNF-α-mediated suppression of Cyp17 promoter activity *(132)*. Together, these data support the concept that TNF-α directly inhibits Leydig cell steroidogenic enzyme gene expression and steroidogenesis, which ultimately contributes to the global reproductive failure associated with chronic inflammation and sepsis. Unlike IL-1, a definitive role for TNF-α under normal physiological conditions has not been established.

Interferons

The IFNs are a group of functionally related protein cytokines, which includes three main groups (α, β, and γ), based on their structural relationships and major cellular sources: IFN-α is produced by monocyte and macrophages, IFN-β is produced by fibroblasts and epithelial cells, and IFN-γ is principally produced by T-lymphocytes *(133)*. The best known effects of IFNs are their antiviral, antiproliferative, and immunomodulatory actions. Both IFN-α and IFN-γ inhibit testosterone production in primary cultures of porcine Leydig cells *(134,135)*. IFN-γ exerts its inhibitory effect on testosterone production at the level of cholesterol transport into the mitochondria. IFN-γ also inhibits the expression of both P450scc and P450c17 *(134)*, similar to the effect of TNF-α on mouse Leydig cells *(126)*. Data indicate that IFNs may contribute to the overall decline in steroidogenic function of patients with viral infections *(136,137)*.

Lymphocyte Inhibitory Factor

The action of leukemia inhibitory factor (LIF) on testicular steroid hormone formation has also been studied. The direct effects of LIF were evaluated on basal and hCG-stimulated testosterone synthesis by cultured, purified Leydig cells isolated from porcine testes. LIF reduced (>60%) hCG-stimulated testosterone synthesis. This inhibitory effect was exerted in a dose- and time-dependent manner. Results indicate that LIF acts by reducing cholesterol substrate availability in the mitochondria. Consequently, LIF action was tested on both StAR and PBR (peripheral benzodiazepine receptor), which is also known to be involved in steroidogenic cholesterol transfer. LIF reduced, in a dose- and time-dependent manner, LH/hCG-induced StAR mRNA

levels. In contrast, LIF had no effect on PBR mRNA expression or PBR binding. This inhibitory effect of LIF on Leydig cell steroidogenesis is probably exerted through an auto/paracrine action of the cytokine. Indeed, by immunohistochemistry, LIF and LIF receptor proteins were identified in Leydig and Sertoli cells but not in other testicular cell types, except for LIF receptor in spermatogonia. Furthermore, the presence of LIF and its receptor in Leydig cells at the neonatal and adult periods suggests that the inhibitory effect of LIF on androgen formation reported here probably occurs in both the fetal and the adult Leydig cell populations during testicular development *(138)*.

Other Cytokines

There is evidence that many other cytokines have direct inhibitory effects on Leydig cell steroidogenesis. IL-2 inhibits gonadotropin-stimulated testosterone production by rat Leydig cells at the level of the P450c17 enzyme, similar to the actions of TNF-α and IL-1 *(139)*. Subcutaneous IL-6 administration in men produced prolonged suppression of serum testosterone levels, without apparent changes in gonadotropin levels, suggesting that this effect of IL-6 may be mediated by direct effects on Leydig cells *(140)*. TGF-β inhibits Leydig cell steroidogenesis at the level of LH receptor number and signaling as well as distal to cAMP production at the level of P450c17 expression *(141,142)*.

Collectively, these experimental and clinical observations predict that in conditions associated with elevated inflammatory mediators, there is a concomitant decrease in serum testosterone levels. One important group of inflammatory mediators, which inhibit Leydig cell steroidogenic function are the proinflammatory cytokines *(3)*. Cytokines are also important for integration of the neuro-endocrine-immune network, which controls testicular function under normal and pathophysiological conditions *(143,144)*. More recently, there has been an increase in interest in the role of ROS in these processes as well.

REACTIVE OXYGEN AND NITROGEN SPECIES

Reactive Oxygen Species

Recognition of an inflammatory or immunogenic signal by both immune and nonimmune cells triggers gene expression for cytokines, adhesion proteins, and enzymes, which produce very low molecular weight inflammatory mediators. The products of these enzymes, the reactive oxygen and nitrogen species, are referred to collectively as ROS and participate in eliminating the infection *(145)*. Important ROS include superoxide anion (O_2^{-}) hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), nitric oxide ($NO^{\bullet}$) and peroxynitrite anion ($ONOO^{-}$). ROS can react directly with and modify cellular macromolecules such as protein, lipids, and DNA. Repair systems exist to correct oxidative damage, but excess and cumulative oxidative damage results in cellular dysfunction and contribute to the pathology of many diseases *(146)*. ROS are produced continuously in cells as the byproducts of mitochondrial and microsomal electron transport reactions and other metabolic processes.

Mitochondrial respiration consumes 85–90% of the oxygen used by cells, and represents the highest potential source of ROS within the cell. The steroidogenic cytochrome P450 enzymes also produce ROS as a byproduct of their catalytic reaction mechanism *(147,148)*. Cellular antioxidant systems that normally protect cells from oxidative damage include superoxide dismutase (SOD), catalase and glutathione peroxidases. Other antioxidant molecules include ascorbic acid, α-tocopherol, β-carotene, retinoic acid, and glutathione. Mitochondrial SOD and redox cycling of GSH and GSSG through the action of glutathione peroxidase affords the cell the greatest protection from ROS generated intracellularly by mitochondria *(149)*. Under normal physiological conditions, ROS generation is controlled and oxidative damage is minimized. However, during oxidative stress such as reperfusion following ischemia/hypoxia, inflammation or when cells are exposed to extracellular sources of ROS, the antioxidant protective mechanisms are overwhelmed and result in cellular oxidative damage. Oxidative damage often results in initiation of apoptosis or necrosis of affected cells *(146)*.

The elaboration of reactive oxygen and nitrogen species during inflammation subjects Leydig cells to oxidative stress. LPS has been shown to activate ROS production from testicular macrophages in vitro *(33)*. Exposure to LPS causes oxidative damage to Leydig cells in vivo, resulting in a collapse of the mitochondrial electrochemical gradient ($\Delta\Psi m$), lipid peroxidation, decreased StAR protein, and a precipitous decline in serum testosterone *(55)*. Hydrogen peroxide, a potent oxidant, inhibits steroidogenesis in Leydig cells *(55,150)* and MA-10 tumor Leydig cells *(56,58)*.

Reactive Nitrogen Species: Nitric Oxide

The free radical nitric oxide (NO) is generated through the oxidation of L-arginine to L-citrulline by NO synthases (NOSs), and has been shown to inhibit Leydig cell steroidogenesis. NO is an important regulatory

molecule involved in the control of blood vessel dilation, immune functions, and neurotransmission, in the central and peripheral nervous systems *(151–153)*. NO is a critical mediator of the pathophysiology of septic shock and LPS endotoxemia *(154–156)*. The two constitutively expressed forms of NOS are found in endothelial cells (eNOS) and the brain (bNOS), respectively. The inducible form (iNOS) is characteristically expressed in macrophages, but is also found in a variety of other cell types *(157)*. The complex and overlapping expression patterns of the three forms of NOS have been extensively studied using specific inhibitors and genetic knock-out models. These approaches enable investigation of the relative contribution of specific forms of NOS in physiological processes *(158,159)*.

LPS stimulates the immediate production of NO from eNOS in endothelial cells *(160)* and induces the expression of iNOS in macrophages, resulting in the production of significant quantities of NO *(161,162)*. NO donors reduced testicular androgen production in vivo *(163)* as well as in vitro *(164)*. The NOS enzymes are expressed by several cell types in the normal testis and iNOS in particular is upregulated by LPS-treatment in Leydig, Sertoli, peritubular, and spermatogenic cells as well as a subset of the testicular macrophages *(45,165,166)*. NO has been shown to inhibit Leydig cell steroidogenesis in vivo *(167,168)* and in vitro *(169–171)*. Treatment of immunologically challenged rodents with NOS inhibitors counteracts the decrease in serum testosterone levels *(110,172,173)*.

The mechanism through which NO inhibits Leydig cells is not known. One possibility is through oxidative damage produced through generation of RNS such as peroxynitrite anion *(174)*. Peroxynitrite is the reaction product of NO and superoxide, and is one of the major cytotoxic agents produced during sepsis, inflammation and ischemia/reperfusion *(175)*. Peroxynitrite decomposes to produce the hydroxyl radicals, which are potent oxidants *(176)* and NO enhances ROS mediated toxicity *(177)*. NO plays only a limited role in the regulation of testicular blood flow under basal conditions. However, after hCG treatment, NOS activity appears to be increased, as inferred from increased endothelial NADPH-diaphorase staining, suggesting that NO in this situation is important to increase blood flow and to inhibit leukocyte accumulation *(178)*.

Controversy exists over the exact cellular location of NOS enzymes in the testicular interstitium. Several publications have reported the NOS isoforms are present in adult Leydig cells, and testicular macrophages. Weismann et al. *(179)*, present data, which indicates that NOS is not expressed in Leydig cells, but is expressed to a significant degree in adjacent macrophages. Gerdprasert et al. *(180)* O'Bryan et al. *(109,181)* propose that iNOS is not expressed in resident testicular macrophages, but is only expressed in inflammatory/infiltrating monocyte macrophages, illustrating that, in the rat testes, there is a heterogeneity of testicular macrophages. In the study by Weismann et al. *(179)* subpopulations of macrophages were not separated out, so it is likely in their investigation that mixed populations of macrophages were examined. Interestingly, Wang et al. *(182)* identified truncated NOS that is uniquely expressed in Leydig cells, and named it "testicular neuronal NOS" (TnNOS). Recently, Herman and Rivier (2006) demonstrated that intracerebroventricular (icv)-delivered IL-1 induced a rapid increase in TnNOS *(183)*. However, TnNOS induction evidently did not mediate the icv injected IL-1-induced suppression of testosterone production *(183)*. Further research will be needed to clarify the role of TnNOS in the regulation of Leydig cell function. Whereas the numerous investigations which have assessed the exact cellular location of NOS isoforms in the testes are not in complete agreement, the consensus of these reports is that increased levels of nitric oxide that accompany inflammation and stress, profoundly inhibit adult Leydig cell steroidogenesis, regardless of the source of NO, or the cellular location of the NOS enzymes. Moreover, reactive oxygen and nitrogen species, produced during postischemic reperfusion of the testis in experimental models of testicular torsion, have been implicated as causing destruction of the seminiferous epithelium and germ cell apoptosis *(184)*. Together, these studies demonstrate the importance of ROS and NO as mediators of the pathophysiological consequences of aberrant immune–endocrine interactions in the testes.

PROSTAGLANDINS

PGs are ubiquitous substances that initiate and modulate cell and tissue responses involved in physiological processes such as platelet aggregation, renin release, and inflammation. Their biosynthesis has also been implicated in the pathophysiology of cardiovascular diseases, cancer, and inflammatory diseases. They are considered local hormones or autocoids because they act in a paracrine or autocrine fashion. Their activity is limited to the site of action by their short half life and also by the fact that they are synthesized on demand and not

stored in tissues. Collectively with thromboxanes, PGs form a group of oxygenated fatty acid derivatives called prostanoids, usually, derived from arachidonic acid. These oxygenated metabolites are also known as eicosanoids because they originate from a 20-carbon (eicosa) polyunsaturated acid. Prostanoid synthesis occurs in three stages: hydrolysis of arachidonic acid from phospholipid precursors catalyzed by a phospholipase A2; Oxygenation of arachidonic acid producing PGG2 and later on, hydroperoxidation of PGG2'S 15-hydroperoxyl group into PGH2. These two reactions (oxygenation and peroxidation) are catalyzed by the closely related isozymes and PG endoperoxide H synthase-1 and synthase-2 (PGH2 synthase). Conversion of PGH2 into biologically active end products: PGD2; PGE2; PGF2-, PGI2 (prostacyclin) and thromboxane A2; catalyzed by various specific synthases (isomerases) or reductases. Prostanoids are involved in signal transduction pathways activated by proinflammatory mediators. There are two cyclooxygenase (COX) isoforms, which differ mainly in their pattern of expression. COX-1 is expressed in most tissues, whereas COX-2 usually is absent, but is induced by numerous physiologic stimuli. COX-1 is involved in homeostatic functions while COX-2 is implicated in various pathological processes such as inflammation and cancer *(185–187)*.

Arachidonic acid metabolism by COX results in the production of prostanoids which inhibit steroidogenesis and promote inflammation, whereas the lipooxygenase metabolism results in the production of prostanoids which promote steroidogenesis *(188,189)*. PGE2 inhibits steroidogenesis and 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and 5-hydroxyeicosatetrenoic acid (5-HETE) promote steroidogenesis *(190)*. Earlier studies demonstrated that PGs inhibit Leydig cell steroidogenesis at the level of cholesterol transfer to the mitochondria *(191–193)*. More recently studies demonstrated that COX-2 exerts a tonic inhibition on Leydig cells through suppression of StAR expression. Selective inhibition of COX-2 causes a significant increase in LH/hCG or cAMP-stimulated StAR expression. The mechanism through which COX-2 exerts its inhibition on StAR is not known, but PGF2α, a product of COX-2, is known to potently inhibit StAR and steroid synthesis *(194,195)*. COX-2 is upregulated by inflammatory stimuli signaling through NF-kB. NF-kB is sensitive to activation by inflammatory stimuli including proinflammatory cytokines, NO, and ROS *(196–198)*. Thus, a self-perpetuating cycle is established when Leydig cells are confronted with inflammatory stimuli—ROS, NO, and/or cytokines. Each of these agents can activate NF-kB, which in turn will upregulate COX-2 expression, resulting in increased PG synthesis, a byproduct of which is more ROS, further increasing NF-kB and COX-2 and PGs *(199)*.

COX-2, AGING, AND IMMUNE DYSREGULATION

ROS and RNS are widely implicated in the inflammatory process. However, mechanistic information is not readily available on the extent to which ROS/RNS contributes to the proinflammatory states of the aging process. The observed age-related upregulation of NF-kB, IL-1β, IL-6, TNF-α, COX-2, and iNOS suggested the "Inflammation Hypothesis of Aging," which supports the molecular basis of the inflammatory process as a plausible cause of the aging process *(199)*. Acute inflammatory assault on Leydig cells mimics many of the features of aging. Dysregulation of COX-2, elevated production of NO, elaboration of cytokines, and reactive oxygen products, all accompany inflammatory responses, result in inhibition of Leydig cell function, and manifest during aging. Moreover, free radicals are produced when PGG2 is converted to PGH2. Macrophage function is altered with aging resulting in uncontrolled production of PGs and over expression of COX-2 *(200–202)*. Leydig cells senesce with age and macrophages become hyperactivated, which suggests that during aging another feature of Leydig cell interactions with macrophages is the uncontrolled elaboration of inhibitory factors, which likely contribute to steroidogenic senescence.

COX-2 expression and activity are increased as a function of aging in Leydig cells, and it has been proposed that the age-related decline in testosterone may be because of this increase in COX-2, resulting in the inhibition of StAR protein expression *(100,203,204)*. There is an age-related increase in COX-2 owing to increased NF-kB resulting from increased ROS production *(205)*, and age-related increases in ROS can directly increase COX-2 catalytic activity without altering COX-2 expression levels *(206)*. Macrophages from old mice produce more PGE2 than those from young mice, contributing to the dysregulation of the immune and inflammatory responses with age *(202)*. These findings have significant implications for age-associated immune and inflammatory dysregulation as well as the development of preventive and therapeutic strategies against them *(207)*.

During aging, testicular macrophages retain their close morphological association with Leydig cells, but

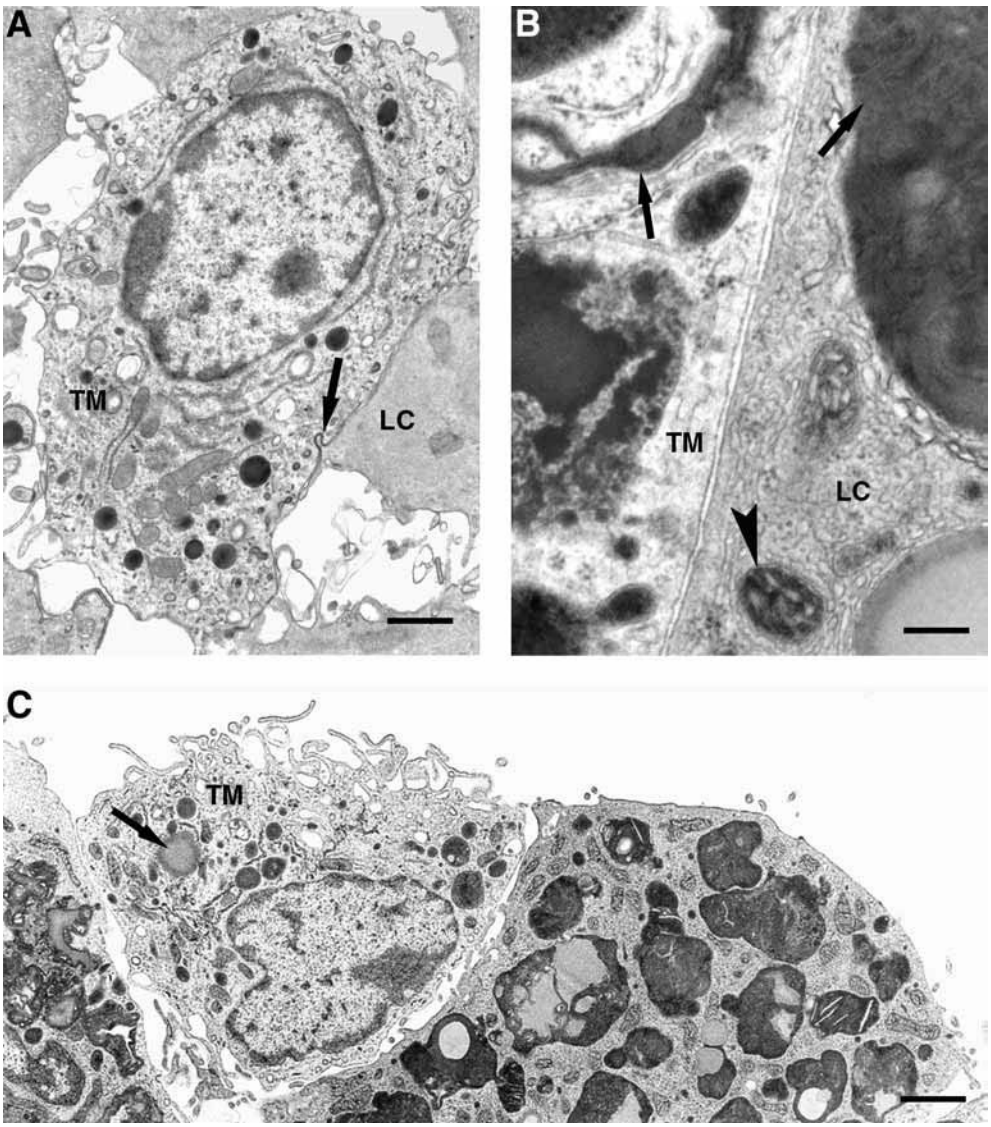


Fig. 2. Digitations that extend from Leydig cells to macrophages are lost with age, but close physical association remains intact. Lipofuscin granules and cytoplasmic lipid vacuoles accumulate in aged cells. **(A)** Adult young mouse. The testicular macrophage (TM) contains lysosomes, mitochondria, and profiles of the rough endoplasmic reticulum. Several small vesicles are also seen. Microvilli protrude from the cell into the intercellular spaces. The free surface in contact with neighboring Leydig cells (LC) is quite smooth and lacks microvilli. A digitation (arrow) is evident. Scale bar = 1.10 µm. **(B)** Aging mouse. A TM and a LC are seen. The plasma membranes retain the arrangement observed in the adult young mice. Coated vesicles are not present in the cytoplasm of the TM. The mitochondria (arrowhead) of the LC are more electron-dense than those found in young adult mice. Lipofuscin granules can be observed both in the TM and the LC. The arrows indicate the lipofuscin granules. Scale bar = 0.26 µm. **(C)** Aging mouse. The TM shows a cytoplasm more lightly stained than that of neighboring LC. Lipid vacuole (arrow). Several lipofuscin granules are present in the Leydig cells. Scale bar = 1.40 µm (Reprinted with permission from ref. *208*).

the cytoplasmic interdigitations are lost *(208)*. Figure 2 demonstrates the age-related loss of interdigitations between Leydig cells and macrophages and the accumulation of lipofuscin granules in both Leydig cells and macrophages. Aging testicular macrophages also acquire lipofuscin granules similar to those observed in aged Leydig cells *(208)*. Lipofuscin is a brown-yellow, electron-dense material, which accumulates with age in postmitotic cells. Lipofuscin accumulates because it can neither be degraded nor be exocytosed from cells.

Lipofuscin is believed to arise because of reduced lysosome activity and cumulative oxidative damage. Mitochondria are implicated as the source of ROS responsible for lipofuscinogenesis, and cells with intense steroidogenic activity generate additional ROS as byproduct of steroid biosynthesis. Damaged mitochondria are

often observed in lipofuscin loaded cells, and altered mitochondria have described in Leydig cells and macrophages from aged rats, mice, and humans *(208)*. In addition to lipofuscin granules, the presence of lipid vacuoles in the cytoplasm of aging macrophages could be associated with alterations in macrophage cholesterol metabolism. Testicular macrophages produce 25-hydroxycholesterol, which is transferred to adjacent Leydig cells and enters the steroidogenic pathway. Thus, it is likely that in the aging testes changes in functional interactions between Leydig cells and macrophages affects macrophage cholesterol metabolism as well as Leydig cell steroidogenesis, and results in the accumulation of lipid vacuoles in the macrophages. Moreover, lipofuscinogenesis is associated with increased NO production and elevated production of cytokines, which would further contribute to the inhibition of Leydig cell function and may be an important mediator in the decline of steroidogenesis with age. The loss of digitations with age may further contribute to decrease steroid production by Leydig cells, by preventing the immediate transfer of 25-hydroxycholesterol from macrophages to the steroidogenic mitochondria of Leydig cells.

CONCLUSIONS AND PROSPECTS FOR THE FUTURE

The discovery of the unique, intimate association between Leydig cells and testicular interstitial macrophages spawned an era of active research into the nature and significance of immune–endocrine interactions in the control of male reproductive function. Whereas much remains to be learned, decades of research have defined the role of immune regulation of Leydig cell function during their development, in the healthy normal adult, and during inflammation and infection. Often the same factors will have trophic or palliative effects in the immature, developing, and noninflamed testis and profoundly inhibitory effects during times of sickness and ill health. The obvious importance of immune–endocrine interactions in the control of Leydig cell function is unequivocal. The new frontier in this field addresses the role of immune-dysregulation during aging and will examine the contribution of aberrant immune influences which contribute to the decline in steroid biosynthesis, resulting in the male andropause.

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22 Activins and Leydig Cell Development, Differentiation, and Disease

Gail P. Risbridger, PhD and Christopher Butler, PhD

SUMMARY

During development and differentiation of many tissues and organs, activins are potent stimulatory or inhibitory factors, but generally during Leydig cell development they are inhibitory. Thus, the decline in activin bioactivity at puberty, corresponds with the emergence of the adult Leydig cell population and differentiation from Leydig cell precursors. Once established, activins inhibit steroidogenesis and oppose or balance luteinizing hormone stimulation. Therefore, together with other local regulating factors, activins are integral to normal Leydig cell function.

During disease and particularly tumor formation in several tissues and organs, activins are growth inhibitory. In order to promote tumorigenesis, cells become resistant to activins as they do to transforming growth factor-β. Resistance to activins could occur through inactivating mutations of receptors, but might also involve activin regulatory proteins, such as inhibin. Little is known about the role of activins in Leydig cell tumors, but the reports of Leydig cell inhibin implicate inhibins as key antagonists of activins, which, when present, might lead to loss of response to activin and hence to Leydig cell disease. Together with their known roles in immune suppression and angiogenesis, activins might modify the systemic environment to further favor Leydig cell disease.

Key Words: Activin; differentiation; inhibin; Leydig cells; tumors.

INTRODUCTION

Activins and inhibins are members of the transforming growth factor (TGF)-β superfamily of signaling molecules, which also includes TGF-β1–3, bone morphogenetic proteins, growth and differentiation factors, Mullerian inhibiting substance (MIS), Nodal, Lefty1, and Lefty2 *(1)*. First identified as regulators of follicle-stimulating hormone secretion, activins were shown to be essential regulators of diverse systems in physiology, with effects on somatic growth, cell proliferation and apoptosis, branching morphogenesis, inflammation, and reproduction *(2–11)*.

Activins and Inhibin

Activins and inhibins are glycoproteins made up of either two β-subunits (Activins) or an α-subunit with either a βA or βB-subunit (Inhibin A or B). There is one α-subunit and there are five activin β subunits; βA, βB, βC, βD, and βE. The activin βA and βB subunits and their homo-/heterodimers Activin A (βAβA), Activin B (βBβB), and Activin AB (βAβB) are well-characterized. To date, the βD subunit has only been found in *Xenopus laevis*, in which it acts as a mesoderm-inducing factor *(12)*. The other two related activin subunits, located in mammalian cells, are activin βC and βE (*see* ref. *13* for review).

Activin subunits are initially produced as glycosylated preprohormones consisting of a signal sequence, a prodomain of varying size and a mature C-terminal segment; this precursor then dimerizes with another subunit. Post-translational modification includes cleaving of these prohormone dimers to form the mature dimeric protein. As with other members of the TGF-β family, activins signal through a serine/threonine kinase pathway utilizing two receptors—activin type I and activin type II *(14)*. Heterodimeric receptor complexes are formed and signaling is transduced by phosphorylation of Smad proteins. Further interaction by Smad proteins with transcription factors leads to altered gene expression *(15)*.

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The Activin Family

Activin A, B, and AB, have similar, overlapping functions, but specific responses of each of the homo and heterodimers are implied from transgenic mouse studies. For example, when the activin βB-subunit was used to substitute for the βA-subunit, it was able to rescue the βA null cranio-facial phenotype and neonatal lethality, but the mice had additional phenotypes including postnatal growth delay and increased mortality *(16)*. Thus somatic growth and survival requires both βA and βB as neither subunit can completely compensate for the lack of the other *(5)*. In addition, tissue-specific expression patterns shown by activin receptors, accessory, and binding proteins suggest differential action of each activin isoform *(17)*.

Inhibins

An inhibin specific receptor signaling pathway was postulated, but evidence for this is not forthcoming. Instead inhibins appear to act by blocking the effect of activins. Inhibin binds the activin type II receptor (ActRII), but prevents recruitment of the activin type I receptor and induction of the signaling cascade *(18,19)*. Inhibin's binding affinity for ActRII is enhanced by the coreceptor molecule betaglycan, which confers sensitivity to inhibin on cells previously unable to respond to it *(20)*.

ACTIVINS AND INHIBINS IN DEVELOPMENT, DIFFERENTIATION, AND DISEASE

Development

Activins play important roles in many developmental processes and across a range of species. In *Xenopus*, activins are required for normal mesoderm development and axis formation *(21,22)*. Critical for neural crest-cell development and craniofacial development in zebrafish, activins are also important in craniofacial development, eyelid closure, and gestation in mice and humans *(23–26)*. In contrast to the important functions of activins in development, inhibins do not appear to play a major role in the developing mammalian embryo. Mice homozygous for the null allele of the inhibin α-subunit develop normally, but the adults are infertile and rapidly develop gonadal tumours of high penetrance. Thus, inhibin-α was reported as "the first secreted protein to have tumour-suppressor activity" *(27)*.

Effects on Proliferation and Cell-Cycle Regulation

Activins have proliferative and antiproliferative actions in many tissues, although, antiproliferative effects are more frequently observed. Activin A promotes proliferation of lung and vascular smooth muscle cells and premyocardial cells *(28–30)*. In contrast, activin A is growth inhibitory in prostate cancer and B-cell leukemia cells, as well as vascular endothelium, vascular smooth muscle, fibroblasts, hepatocytes, liver cells, and fetal adrenal cells. Growth inhibition by activin commonly involves cell cycle arrest in the G1 phase or induction of apoptosis by caspase activation (*19,31–35*, *see* ref. *36* for review).

Differentiation

Activin plays a prominent role in differentiation of various cell types. During development of the *Xenopus* embryo, differences in the concentration gradient of activin A are believed to direct the differentiation of cells into different lineages *(37–39)*. In mammals, activin promotes differentiation of dI3 interneurons in the spinal neural tube and acts as a differentiation factor in kidney development *(40,41)*. Differentiation of fetal liver cells expressing PDX-1 toward a β-cell pancreatic phenotype is regulated by activin A *(42)*. In combination with *Wnt4*, activin acts synergistically to promote differentiation of mouse embryonic stem cells *(43)*; with other factors, activin induces mouse embryonic stem cells to differentiate to insulin-producing cells and is necessary for induction of haematopoietic mesoderm markers *(44,45)*. In contrast, activin A transiently inhibits proliferation and differentiation of limb muscle precursors in chick embryo limb-buds and inhibits adipogenesis in 3T3-L1 preadipocytes *(46,47)*.

Disease

Immunosuppressive Effects

Members of the TGF-β growth factor family, including TGF-β and activins, regulate the immune system. Inhibin facilitates, and activin inhibits, the TGF-β-mediated immunosuppression of thymocytes stimulated by concanavalin A (Con-A) *(48)*. Activin A concentrations in the bloodstream are acutely responsive to inflammatory challenge in postnatal life and activin A might be a significant component of the innate immune system *(49)*.

Angiogenesis

Blood vessel formation or angiogenesis is a crucial process in both development and disease and activins regulate angiogenesis in both circumstances. Activin A inhibits vascular endothelial cell growth and angiogenesis in vivo and endothelial cell proliferation in xenograft tumors *(50–52)*. Follistatin, an activin-binding protein, induces proliferation of endothelial cells *(53)*. These

results suggest activin suppresses tumor development by restricting blood vessel growth *(36)*. Conversely, activin A stimulates inflammatory corneal angiogenesis by increasing vascular endothelial growth factor levels and stimulates vascular endothelial growth factor in hepatocellular carcinoma *(54,55)*.

Cancer

The importance of activin in endocrine cancers is implied by the number of activin regulatory proteins such as follistatins and inhibins associated with tumors as well as loss of function mutations of the activin receptors. Follistatin is overexpressed in hepatocellular carcinoma and therefore, prevents activin action by decreasing its local bioavailability *(56)*. Both follistatins and Smad7 regulate activin action by a negative feedback loop and their own expression is stimulated by activin *(36)*. The ActRII is commonly mutated in the high frequency microsatellite instability form of colon cancer, where it is thought to contribute to tumorigenesis by disrupting access to alternative TGF-β effector pathways, and a loss of function mutation of *ActR-IB* was identified in human pituitary tumours *(57–59)*.

Metastasis

The effects of activins on metastasis are varied, and activins are both positively and negatively correlated with metastasis. Thus, in esophageal carcinoma, Activin A expression is positively associated with tumor aggressiveness and lymph node metastasis *(60,61)*. Conversely, Activin A suppresses neuroblastoma xenograft tumor proliferation without affecting lung metastasis *(51)*. Finally, activin increases NCAM expression which itself is negatively correlated with tumor progression and activin is expressed in poorly metastatic melanoma cells but is absent from highly metastatic cells *(62,63)*.

Inhibin in Cancer

Inhibin is a tumor suppressor with gonadal specificity in mice *(27)*. Inhibin α-subunit expression is downregulated in human prostate cancer tissues and cell lines as a result of hypermethylation of the promoter region of the gene or loss of heterozygosity *(64,65)*. Conversely, inhibin is upregulated in ovarian cancer and serum inhibin levels are used as a diagnostic for detecting ovarian cancer and recurrence in granulosa cell tumours *(66,67)*. These data sets are contradictory and paradoxical, but a plausible explanation, based on the emerging evidence of a dual role of TGF-β in breast cancer, is that the *INH-A* gene has dual roles and is both tumor suppressive and prometastatic in a single cell lineage *(68,69)*. Ongoing studies in this laboratory aim to provide proof of this concept. The preceding introduction on activins and inhibins briefly reviews their significant roles in development, differentiation, and disease of a wide range of cells and tissues and in doing so, predicts their role in Leydig cell development and disease.

ACTIVIN AND INHIBINS IN LEYDIG CELL DEVELOPMENT AND DIFFERENTIATION

Development and Differentiation in Normal Leydig Cell Populations

The function of activin as a growth and differentiation factor for Leydig cells is evident during development and differentiation. During fetal life, the Leydig cell population emerges, synthesizes steroids necessary for sexual differentiation, remains in neonatal life, and declines thereafter *(70)*. At puberty a second generation of Leydig cells develop, mature and give rise to the adult Leydig cell population that synthesises androgens within the normal adult male testis *(71,72; see* Fig. 1). At each of these stages there is expansion of the Leydig cell populations and differentiation resulting in steroidogenesis. Although, the gonadotrophin luteinizing hormone (LH) has an essential role in this process, there is an additional level of control within the testis by growth factors, such as the activins. Although, the gonadotrophins stimulate Leydig cell proliferation and differentiation, activins inhibit these processes and must be regarded as local inhibitors that oppose the overall stimulation from the pituitary gland.

Inhibin in Leydig Cells

At the time of writing a previous review on Leydig cells and activins/inhibins, both ligands were believe to signal through specific receptors *(73)*. It was in this context, that activin and inhibin were regarded as dual opposing regulators of Leydig cell function. With the benefit of hindsight and the failure to conclusively demonstrate the presence of an inhibin receptor, those observations can now be reinterpreted with the contemporary view that inhibin functions simply as an antagonist for activin ligands *(74–76)*. It is one of several proteins, which are proven to modify activin ligand bioactivity; other factors include follistatin and BAMBI *(77)*.

Activin and the Leydig Cell

Activin Action in Fetal Leydig Cells

During fetal Leydig cell development, all of the subunit proteins for activin and inhibin proteins are expressed in the rodent and human testes *(78,79)*. Activins are important regulators of gonocyte differentiation at this

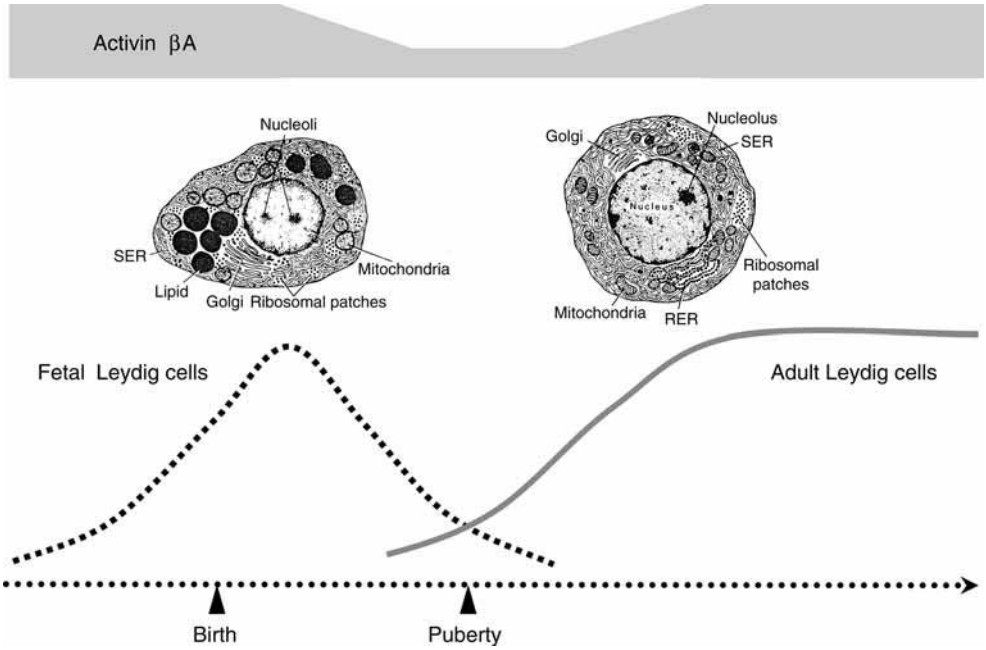


Fig. 1. Correlation of Leydig cell development and activin levels. During fetal Leydig cell development activin βA levels are high, but decrease when the fetal population regresses. Decline in activin production by immature Leydig cells is coincides with proliferation and differentiation of immature cells to mature Leydig cells of the adult testes.

time, but also appear to be produced and act on interstitial cells which include fetal Leydig cells *(80,81)*. The fetal population of Leydig cells persists in neonatal life and activins regulate steroidogenesis. Specifically, activins inhibit testosterone production by interstitial cells isolated and cultured from neonatal testes, whereas inhibin stimulates LH regulated steroidogenesis; inhibitory effects of activins on Leydig cell steroidogenesis also occur in other species such as pig *(81,82)*. Now it is known that this is most likely because of antagonism of endogenous activin ligand synthesis. Yet, very little is known about the production of activin ligands by interstitial cells, because of the limited availability of assays to detect and measure dimeric activins, especially activins B and AB *(83–85)*. One can only postulate that a critical balance between these proteins would determine the steroidogenic response to gonadotropin and maintain homeostasis.

Pubertal Development of the Adult Leydig Cell

During puberty, when a second generation of Leydig cells emerges from the precursor cells in the interstitium, proliferation, and differentiation are critical processes. Because activins are generally growth inhibitory, it is not surprising that activin production by immature Leydig cells declines in the pubertal rodent testis coincident with proliferation and differentiation of the immature cells to mature Leydig cells of the adult testes *(83,84,86,87)*. Once the adult Leydig cell generation is established, activin appears to remain as an effective negative regulator of testosterone synthesis, but whether or not it holds proliferation of the adult population in check is unclear *(81,88)*.

The capacity to regenerate the adult population of Leydig cells is evident from studies using ethane dimethane sulphonate, which selectively eliminates the adult Leydig cell population *(89)*. When administered to rodents, ethane dimethane sulphonate destroys adult Leydig cells and proliferation and differentiation of Leydig cell precursors is activated giving rise to a new population of functionally active Leydig cells. This experimental model was used to study the expression of inhibin and activins *(87)*. The authors concluded that adult Leydig cells differentially modulate and express these genes, further implicating activins as critical regulators of adult Leydig cell homeostasis.

Regulation of Leydig Cell Proliferation

As well as activin, TGF-β inhibits Leydig cell proliferation restricting Leydig cell growth until puberty when it too declines *(90–92)*. This raises the question of why similar factors perform the same function. Is the action of LH so profound that several factors are required to act in synergy to restrain its action and/or is an inherent redundancy built into the system because it is so important to modify LH action on the Leydig

cells to prevent the LH-induced onset of puberty? Furthermore, do activins and TGF-β as well as other members of the superfamily such as MIS, have the same inhibitory effect? Is the potency of each of the ligands similar? Do they act sequentially or simultaneously? The answers to these questions remain unknown and are difficult to resolve when ligands such as activin AB and to a lesser extent activin B, are restricted in their availability. Regardlessly, an important difference between activins and TGF-β lies in the processing of the proteins. Upon secretion, activin is a bioactive dimer, whereas TGF-β is secreted in latent form requiring proteolytic cleavage to become active. This fundamental difference between the ligands might explain the presence of a range of antagonists or binding proteins for activins, such as inhibin and follistatin.

Regulation of Activin Action

The inhibin α-subunit is expressed in adult Leydig cells and together with the α-subunits it is possible that dimeric inhibin is produced together with activin *(83,84)*. Although, inhibin was measured by immunoassay in cultures of Leydig cells, there is some suggestion that the assay measured α-subunit proteins rather than dimeric biologically active inhibin secreted by Leydig cell tumors *(93,94)*. Dimeric inhibin blocks activin from binding to its receptor and if produced, the net effect of activin would be dependent upon the balance of the two ligands. Follistatin (FS) is another regulator of activin bioactivity being a binding protein with an affinity for activin, similar to that of its receptor. FS immunoreactivity is localized to interstitial cells, but mRNA was not detected by *in situ* hybridization or reverse transcriptase-polymerase chain reaction and might be synthesized elsewhere in the testis *(95,96)*. Nevertheless, overexpression of follistatin results in variable degrees of Leydig cell hyperplasia *(97)*, consistent with neutralization of the growth inhibitory effects of activins. Another newer member of the FS family, FS-like 3 (FSLT3) is also highly expressed in the testis and FS-like 3 protein was localized to Leydig cells *(98)*. Although, its biological functions do not exactly overlap with FS, Leydig cell hyperplasia also occurs in transgenic mice overexpressing FSLT-3, consistent with blockade of activin action on Leydig cells. In both the FS and FSLT-3 overexpressing mice, the concomitant disruption of spermatogenesis and disruption of tubular-interstitial cell reciprocal signaling must also contribute to the Leydig cell phenotype.

Activins and members of the TGF-β superfamily signal through activation of specific receptors and the Smad intracellular signaling pathway. Receptors for type I and II activin/TGF-β receptors and SMAD proteins are present in Leydig cells *(84,99–101)*, although much of what is known about SMADs in Leydig cells relates to their role as mediators of MIS, another member of the TGF-β superfamily of growth factors *(102–104)*. Overexpression of Smad 4, a common mediator of TGF-β, activin, and bone morphogenetic proteins signaling, causes germ cell ablation in some, but not all transgenic mice resulting in fertile and infertile groups. The infertile show variable degrees of Leydig cell hyperplasia, whereas the fertile group do not, suggesting the Leydig cell hyperplasia is secondary to germ cell ablation *(105)*.

LEYDIG CELLS IN DISEASE

Leydig Cell Tumors and Cancer

Inhibin is used as a marker of some types of ovarian cancer, although it was first described as a tumor suppressor with gonadal and adrenal specificity *(27)*. Activin/inhibin subunits and activin receptors are expressed in Leydig tumor cells in humans, rodents, dogs, and stallions *(84,101,106–110)*. The precise forms of inhibin expressed by Leydig cell tumors are unclear *(109)*. Most studies detect the proteins by immunolocalization and some studies measured the ligands by assay technique, which detects both dimeric inhibin and free subunit proteins (that might be biologically inactive). Nevertheless, the immunolocalization of inhibin α-subunits appears to be useful for the identification of Leydig cells in sex cord-stromal cell tumors in several species *(106,111–115)*. Inhibin expression in Leydig cells was successfully used to differentiate testicular sex cord-stromal tumors from germ cell tumors *(114)*. Islands of Leydig cells were identified by inhibin staining in an ovarian yolk-sac tumor *(111)*.

Tumour Progression

Despite the utility of inhibin as a marker for Leydig tumor cells, very little is known about the role of these proteins in the initiation and progression of tumorigenesis. Inhibin-deficient transgenic mice develop sex cord-stromal tumors, demonstrating inhibin is a critical negative regulator of gonadal stromal cell proliferation and the first secreted protein identified to have tumour-suppressor activity. This experimental evidence does not implicate inhibin as oncogenic or prometastatic. The mechanism underlying the role of inhibin and activin in Leydig cell tumors bears remarkable similarity to the situation in ovarian and more recently prostate cancer. Specifically, the idea that inhibin and activin are tumor suppressors is equivocal because the data are

inconsistent. Why does deletion of the inhibin α-subunit gene promote ovarian tumors, but inhibin is used and has been validated as a marker of recurrent ovarian cancer in women? Why is inhibin gene expression lost in men with prostate cancer (because of loss of heterozygosity and promoter hypermethylation), yet upregulated in men with advanced cancer? Our current hypothesis proposes inhibin is both tumor suppressive and prometastatic, similar to TGF-β in breast cancer *(69)*. Whether or not inhibin has dual roles in Leydig cell tumorigenesis, remains unexplored. Finally, the interaction with other regulatory proteins (e.g., FS and BAMBI) in Leydig cell tumors is unknown and fertile ground for investigation.

CONCLUSIONS

Activins and inhibins are essential regulators of diverse organ systems in physiology, with effects on growth and differentiation, as well as disease and their actions are observed in a range of species. During development and differentiation, activins can be potent stimulatory of inhibitory factors, but during Leydig cell development they are generally inhibitory. The presence of two waves of Leydig cell development giving rise to fetal and adult Leydig cell populations correlates with activin levels. Thus, the decline in activin bioactivity at puberty, corresponds with the emergence of the adult Leydig cell population and differentiation from Leydig cell precursors. Once established, activin inhibits steroidogenesis, and opposes or balances LH stimulation. Regulation of LH stimulation of Leydig cells is very tightly controlled by both activins and TGF-β. It is not known why this redundancy of control mechanism occurs. During disease and particularly tumor formation in several tissue and organs, activins are growth inhibitory and in order to promote tumorigenesis, cells become resistant to activins as well as TGF-β. Resistance to activins could occur through inactivating mutations of receptors, but might also involve activin regulatory proteins such as inhibin. Little is known about the role of activins in Leydig cell tumors, but the reports of Leydig cell inhibin implicate inhibins as key antagonists of activins that, when present, might lead to loss of response to activin and lead to Leydig cell disease. Inhibin acts as a marker for Leydig cell tumors and successfully distinguishes sex-cord stromal tumors from germ cell tumors. Inhibin is downregulated in early cancers and inhibin-null mice develop gonadal tumours. However, inhibin is often upregulated in ovarian cancers and advanced prostate cancer. A new proposal suggests that, like TGF-β, inhibin might have dual roles as a tumor suppressor in early stage cancer and metastatic enhancer in late stage cancers. Whether or not inhibin has dual roles in Leydig cell tumorigenesis remains unexplored.

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23 Transcription Factors as Regulators of Gene Expression During Leydig Cell Differentiation and Function

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SUMMARY

Transcription factors are at the center stage of several cellular processes, in which they play essential roles as receivers, interpreters, and conveyers of numerous extracellular signals. These signals are the cues to which cells must respond and transcription factors are indispensable to translate these signals into a genomic response. Depending on the signal, different transcription factors will be solicited leading to unique combinatorial interactions, or codes, required for the activation of specific sets of genes ultimately, triggering cell proliferation, specification, differentiation, function, or death. As for most cell types found throughout the body, testicular Leydig cells have a unique function; they produce the hormones (insulin-like 3 and testosterone) required for fertility and maleness in men. Several signals, such as hormones and signaling molecules, have been identified as important regulators of Leydig cell differentiation and function. Downstream of these signals are transcription factors. The aim of this chapter is to provide a description of the various families of transcription factors, which have been identified as regulators of Leydig cell gene expression and function.

Key Words: DNA binding; hormone; Leydig cells; nuclear proteins; promoter; steroidogenesis; testis.

INTRODUCTION

Cell differentiation is a critical process, which accounts for the variety of the cell types that arise during development. Transcription factors play important roles in this process. They receive and interpret diverse signals and translate this information into molecular signatures through their ability to interact and form codes that are unique for the activation of specific sets of genes ultimately, specifying the fate of a cell or a particular response. Identifying these factors and elucidating their mechanism of action is of much importance for our understanding of cell differentiation and function in all tissues. This includes Leydig cells, which are the main androgen-producing cells of the mammalian testis.

The regulatory signals, which control the differentiation and function of both populations of Leydig cell have been well-characterized *(1,2)*. However, the molecular mediators/transcription factors downstream of these signals are not yet fully understood. Besides serendipity, several strategies exist to identify these transcription factors. One involves the characterization of promoter region of genes that are specifically expressed in the tissue or cell type of interest such as Leydig cells. This often leads to the identification of transcription factors that, in addition to regulating expression of the gene of interest, turn out to be involved in the specification and/or the differentiation of that particular cell type. This classical approach has been successfully used for decades in numerous tissues and cell types, including Leydig cells.

TRANSCRIPTION FACTORS EXPRESSED IN LEYDIG CELLS

As mentioned earlier, Leydig cell differentiation and function requires the well-orchestrated action of several hormones and signaling molecules produced by different endocrine and paracrine/autocrine cells throughout the body. In response to these signals, a multitude of genes will be expressed, but they must be expressed at the right place and at the right time to produce the distinctive characteristics of Leydig cells.

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The mechanisms controlling spatio-temporal gene expression constitute the basis of most biological processes in health and disease. The key to deciphering these mechanisms resides in the understanding of the transcription factors, which are involved in directing tissue-specific and hormonally regulated gene expression.

Transcription factors are nuclear proteins that regulate tissue-specific gene expression by acting as either positive or negative regulators of gene expression. Nearly 3000 transcription factors have been identified in eukaryotes and they have been classified based on similarities in the amino acid sequence of their DNA-binding domains *(3)*. These include the following superclasses: basic domains (b-helix–loop–helix [HLH], b-leucine zipper [ZIP], bHLH-ZIP, bHSH, and so on), zinc fingers (ZnF) (nuclear receptors, GATA, specificity protein [Sp]1, and so on), helix–turn–helix (HTH; homeobox), and β-scaffold (nuclear factor [NF]-κB, signal transducer and activator of transcription [STAT]). This classification has been used in the following sections to provide an overview of the transcription factors that have been identified in Leydig cells (Table 1).

The Superclass of Basic Domains

This superclass of transcription factors is characterized by a DNA-binding domains made up of basic amino acids, which form an α-helix when interacting with DNA. This domain is often found next to a dimerization interface; members of this superclass must dimerize in order to bind to DNA. The various dimerization domains (ZIP, HLH, and so on) were used to divide the basic domain superclass into classes. Members of these classes have been reported in Leydig cells where they have been shown to regulate gene transcription.

The Class of bZIP Factors

The ZIP domain is a DNA-binding domain found in numerous transcription factors. ZIPs have a repetitive pattern in a stretch of 35 residues that consists of a leucine followed by six other residues to form a heptad. This class of transcription factors is further subdivided into several families such as the AP-1, cAMP response element-binding protein (CREB), and CCAAT/enhancer-binding protein (C/EBP) families.

AP-1 Family: Jun and Fos

The AP-1 family is mainly made up of the Jun (*c*-Jun, JunB, JunD) and Fos (*c*-Fos, FosB, Fra-1, Fra-2) subfamilies. Fos members can form heterodimers with Jun proteins, whereas Jun members can form either homo- or heterodimers. They are ubiquitously expressed and have been shown to be involved in a wide variety of cellular processes, including cell proliferation, cellular and viral gene expression, cell death, survival, differentiation, and tumorigenesis *(4)*. Although, AP-1 family members were initially described in Leydig cells several years ago *(5)*, the first target gene for these transcription factors has only been reported recently by Stocco and colleagues *(6)*. They found that the promoter of the gene encoding the steroidogenic acute regulatory protein (*StAR*), a protein essential for the transport of cholesterol from the outer to the inner mitochondrial membrane, contains a functional AP-1 binding site *(6)*.

CREB Family

The CREB family is made up of two widely distributed members, CREB and cAMP response element modulator (CREM), each produced as multiple isoforms *(7)*. CREB and CREM were shown to activate the StAR promoter in Leydig cells by binding to an element located at –85 bp *(8,9)*. Supporting a role for CREB in hormone-induced *StAR* expression, DNA-binding activity of CREB was increased following cAMP stimulation of Leydig cells *(9)*.

C/EBP Family

The C/EBP family is made up of six members: C/EBP-α, -β, -γ, -δ, -ε, and -ζ *(10)*. C/EBP-β is the predominant member present in Leydig cells. Its expression is correlated with the differentiation state of Leydig cells; it is weakly expressed in immature Leydig cells and its expression increases in mature Leydig cells *(11)*. Furthermore, C/EBP-β expression was shown to be upregulated in response to LH suggesting that this transcription factor might be involved in the hormonal regulation of Leydig cell gene expression *(11)*. C/EBP-β was shown to activate *StAR* transcription by binding to two elements (at –113 and –87 bp) *(12)*. C/EBP-β has also been shown to physically interact with the transcription factors SF-1 and GATA-4 leading to further activation of the *StAR* promoter *(12,13)*.

The Class of bHLH Factors

The numerous bHLH proteins are categorized into seven classes based on their expression pattern and their dimerization properties *(14)*. They regulate gene expression by binding to an E-box motif (CANNTG). Leydig cells are likely to express several bHLH factors but thus far, only one has been described. SHARP-2 (Stra13/DEC1) is present in MA-10 Leydig cells where its expression is upregulated in response to gonadotropins *(15)*. No target genes have been identified for this factor in Leydig cells.

Table 1
Classification of Transcription Factors Identified in Leydig Cells

Superclass	*Class*	*Family*	*Transcription factor*
Basic domains	ZIP	AP-1	*c*-Jun
			c-Fos
			Fra-2
		CREB	CREB
			CREM
		C/EBP	C/EBP-β
	HLH	Hairy	SHARP-2
	HLH/ZIP	Ubiquitous	USF1
			USF2
			SREBP
			Spz1
	Helix-Span-helix	Ap-2	Ap-2
Zinc-cordinating DNA-binding domains	Cys4 ZnF of nuclear receptor type	Steroid hormone receptors	GR
			MR
			AR
			ERα and ERβ
		Thyroid hormone receptor-like	RARα, RARβ, and RARγ
			RXRα, RXRβ, and RXRγ
			Nurr77 (NGFI-B)
			Nurr1
			SF-1
			LRH-1
			PPARα and PPARβ
			TR4
			COUP-TFI
	Diverse Cys2His2 ZnFs	GATA	GATA-4
	Cys2His2 ZnFs	Ubiquitous	Sp1
			Sp3
		Developmental/cell-cycle regulators	Egr-1 (NGFI-A)
			GIOT1
			GIOT2
HTH	Homeodomain	Homeodomain only	Arx
			Rhox4
			Pbx1
		Homeodomain with LIM region	Lhx9
β-Scaffold	Rel homology region	Rel/ankyrin	NF-κB
	STAT	STAT	STAT5A
			STAT5B
	Heteromeric CCAAT factors	Heteromeric CCAAT factors	NF-Y

The Class of bHLH-ZIP Factors

Members of this family contain two independent dimerization domains, an HLH and a ZIP, thus providing an additional level of control through dimerization specificity. Four members of this class have been reported in Leydig cells: upstream regulatory factor (USF)1, USF2, sterol regulatory element binding protein (SREBP), and spermatogenic zip (Spz)1; although very little information is available regarding this factor *(16)*.

The widely distributed USF1 and USF2 bind as homo- and heterodimers to the E-box motif CACGTG or CACATG *(17)*. Both factors have been detected in Leydig cell lines *(18)* and in vivo (Pons E and Tremblay JJ, unpublished). So far, USF1/2 have been shown to regulate transcription of the *SF-1* gene *(18,19)*. Consistent with a role in male reproductive function, *USF2*$^{-/-}$ male mice displayed fertility problems, but the exact cause of the defect has not yet been determined *(20)*. SREBP

factors are involved in lipid homeostasis in mammalian cells. In Leydig cells, the *StAR* promoter was shown to be regulated by SREBP1 through transcriptional cooperations with Sp1, NF-Y, and SF-1 *(21)*.

The Superclass of Zinc-Coordinating DNA-Binding Domains

The ZnF DNA-binding motif has been identified in several hundred transcription factors making the ZnF superclass the largest. ZnF motifs consist of small protein domains that fold around a zinc ion that is bound to the Cys or His residues of the finger. Differential use of the two residues gives rise to several types of ZnFs, such as C_2H_2, C_2HC, C_2C_2, C_2HC–C_2C_2, and C_2C_2–C_2C_2. In addition to their role as a DNA-binding module, ZnFs have also been implicated in protein–protein interactions. The vertebrate ZnF factors have been separated into three large classes of which several members have been identified in Leydig cells (Table 1), where they were shown to regulate transcription of several genes (Table 2). In addition, mutations of some of these ZnF transcription factors have been shown to impair Leydig cell development and function.

The Class of C_2C_2 Nuclear Receptor Type

Nuclear receptors have two nonrepetitive C_2C_2 ZnFs. This class of transcription factors is divided into two families: the steroid hormone receptors (ER-α, ER-β, Glucocorticoid Receptor [GR], Mineralocorticoid Receptor [MR], Progesterone Receptor [PR], Androgen Receptor [AR]), and the thyroid hormone receptor-like factors (Thyroid hormone Receptor [TR], RAR, retinoid X receptor (RXR), Vilamin D Receptor (VDR), Peroxisome Prolifererator Activated Receptor (PPAR), SF-1, Nur77, Nurr1, COUP-TFI, and so on). In this class, the N-terminal ZnF makes sequence-specific contact with DNA, whereas the C-terminal ZnF acts as a dimerization interface. Steroid hormone receptors therefore, bind as homo- and heterodimers to a doublet of the consensus sequence AGGTCA organized as direct (→n→), inverted (→n←), or everted (←n→) repeats separated by a variable number of nucleotides depending on the dimerization partner *(22)*. Some members of the second class of nuclear receptors can bind to DNA as dimers (TR, RAR, RXR, VDR, PPAR, Nur77), whereas others, such as SF-1, Nur77, COUP-TF, can also bind to DNA as a monomer. However, this requires a variant DNA element, which contains an additional three nucleotides 5′ of the AGGTCA core sequence. A comprehensive review on nuclear receptors can be found in ref. 22. Of the numerous nuclear receptors found in Leydig cells, SF-1 and Nur77 have been shown to regulate transcription of numerous genes.

The SF-1 Family

Steroidogenic factor (SF)-1, also known as Ad4BP or NR5A1, was the first transcription factor identified as an essential regulator of several genes encoding steroidogenic enzymes *(23,24)*. SF-1 binds to a 9 bp regulatory motif, TCAAGGTCA, found in the promoter region of its target genes, which include StAR *(12,25–27)*, P450 side-chain cleavage enzyme (P450scc) *(28–31)*, 3β-hydroxysteroid dehydrogenase (HSD) *(32,33)*, Cyp17 *(34–36)*, Cyp19 *(37–40)*, Luteinizing Hormone Receptor (LH-R) *(41)*, Prolation Receptor (PRL-R) *(42)*, Müllerian Inhibiting substance (MIS) Receptor Type II (RII) *(43)*, Vanin-1 *(44)*, and Insulin like (INSL) 3 *(45–47)*. An exhaustive description of SF-1 target genes in steroidogenic tissues is provided elsewhere *(48)*.

The in vivo role of SF-1 was evaluated by gene inactivation in the mouse. *SF-1*$^{-/-}$ mice lack adrenal glands and gonads, and display female internal genitalia, including XY individuals *(49,50)*. Moreover, some cases of adrenal failure and sex reversal in human were shown to be caused by mutations in the *SF-1* gene (reviewed in ref. *51*). As *SF-1*$^{-/-}$ mice had no gonads, a Cre-Lox approach was therefore used to generate a Leydig cell-specific *SF-1* knockout *(52)*. In these animals, Leydig cells were present indicating that SF-1 is not required for their specification. Supporting a role for SF-1 in *INSL3* expression in Leydig cells *(45,46)*, testis descent was partially impaired in Leydig cell-specific *SF-1*$^{-/-}$ mice *(52)*. Expression of two other Leydig cell markers, P450scc and StAR, was undetectable, thus, confirming the requirement for SF-1 in the *in vivo* expression of some steroidogenic genes, at least in fetal Leydig cells *(52)*. However, additional *in vivo* experiments, such as a targeted ablation of *SF-1* just before puberty, are required to better define its role in the adult population of Leydig cells.

Another nuclear receptor called liver receptor homolog 1 (LRH-1/FTF/NR5A2) is also found in Leydig cells *(53)* (Martin LJ and Tremblay JJ, unpublished). LRH-1 is highly related to SF-1 and can bind to the exact same sequence as SF-1 (reviewed in ref. *54*). So far, LRH-1 has been shown to activate several promoters just as well as SF-1, including StAR *(55)*, Cyp17 *(55)*, P450scc *(55,56)*, Cyp19 *(53)*, HSD3B2 *(33,55,57,58)*, and INSL3 *(47)*. Therefore, it is likely that LRH-1 and SF-1 could play redundant roles in the transcriptional regulation of several genes in Leydig cell. A definitive answer as to the *in vivo* contribution of LRH-1 to Leydig cell function remains unknown, as *LRH-1*$^{-/-}$ mice die *in utero* at embryonic day 6.5 as a result of severe gastrulation defects *(59)*.

Table 2
Transcription Factors and Their Target Genes in Leydig cells

Transcription factor	*Target gene*[a]	*References*
AP-2	*mLH-R*	*121,122*
GATA-4	*p, mStAR*	*91,19,89*
	r, hCyp17	*86,88*
	hHSD3B2	*33*
	mSF-1	*19*
	mCyp19	*19*
C/EBPβ	*mStAR*	*12,13*
Sp1/Sp3	*mLH-R*	*97*
	m-Vascular endothelial growth factor	*99*
	mPBR	*98*
	r-Scavenger receptor class B type I	*96*
SF-1	*b, r, m, hStAR*	*25,26,12,27*
	b, r, hP450scc	*28,29,30,31*
	b, r, hCyp17	*34,35,36*
	hHSD3B2	*32,33*
	r, hCyp19	*37–39,40*
	rLH-R	*41*
	m, hINSL3	*45,46,47*
	rPRL-R	*42*
	mVanin-1	*44*
	rMIS-RII	*43*
LRH-1	*r, hCyp19*	*53*
	hStAR	*55*
	hHSD3B2	*33,55,57,58*
	hINSL3	*47*
	hP450scc	*55*
	hCyp17	*55*
AP-1 (*c*-Jun/*c*-Fos)	*mStAR*	*6*
Nurr77	*hStAR*	*69* and our unpublished data
	hHSD3B2	*57*
	mHSD3B1	*69*
	rCyp17	*69,70*
	hINSL3	*47*
SREBP	*rStAR*	*21*
CREB/CREM	*mStAR*	*8,9*

[a]The letter preceding the name of the gene refers to the species: b, bovine; p, porcine; m, mouse; r, rat; h, human.

The Nur77 Family

Nur77, also known as NGFI-B, TR3, or NR4A1, is the founding member of the NR4A family of orphan nuclear receptors, which also includes Nurr1 (NR4A2) and Nor1 (NR4A3). NR4A family members are known to be immediate early response genes in several tissues (reviewed in ref. *60*), including hormonally stimulated steroidogenic cells *(57,61–64)*. NR4A family members are known to bind mainly as a monomer to a Nur77 response element (AAAAGGTCA), which is very similar to the SF-1 binding site *(65)*. They have also been shown to bind as homo- or heterodimers to an inverted repeat response element *(66,67)* or to a direct repeat (DR)5 element by heterodimerization with RXR *(68)*.

In the past few years, members of the NR4A family of nuclear receptors, particularly Nur77 and Nurr1, have received increased consideration as novel regulators of basal and hormone-induced gene transcription in testicular Leydig cells. So far, Nur77 was shown to activate several promoters in Leydig cells: *HSD3B2 (57)*, *Cyp17 (69,70)*, *StAR (69)* (Martin LJ, Boucher N, and Tremblay JJ, submitted), and *INSL3 (47)*.

The *in vivo* role of Nur77 has also been assessed by gene targeting in the mouse. Surprisingly, *Nur77*-deficient mice appear normal and are fertile *(71,72)*. At first sight, this would argue against a role for Nur77 in male reproductive function. However, functional redundancy among Nur77 family members could explain this

lack of phenotype *(71,73)*. It is therefore likely that Nurr1 present in Leydig cells *(61)* (Martin LJ and Tremblay JJ, unpublished data) could compensate for the absence of Nur77. In agreement with this, Nurr1 has been recently shown to activate several promoters as well as Nur77 *(57)*. More experiments are required to better define the *in vivo* role of the NR4A family of nuclear receptors in Leydig cell gene expression.

The Class of Diverse C_2C_2 ZnFs

Six GATA factors, GATA-1–6, make this class of transcription factors. They all contain a DNA-binding domain made up of two similar C_2C_2 ZnFs of which the C-terminal is essential for specific binding to the GATA motif (A/T)GATA(A/G). The six GATA factors are expressed in numerous tissues where they play essential roles in cell differentiation, organogenesis, steroidogenesis, and cell-specific gene expression *(74–80)*. Of the six GATA factors, only GATA-4 is found in testicular Leydig cells.

The GATA-4 Factor

GATA-4 is found in both fetal and postnatal Leydig cells *(81–84)* where it was shown to activate several steroidogenic genes (Table 2). These include *StAR, HSD3B2*, *Cyp17*, and *Cyp19 (19,33,79,85–91)*. A direct implication of GATA-4 in steroidogenic gene expression *in vivo* remains to be confirmed because *GATA-4*$^{-/-}$ mice die embryonically because of cardiac defects, before the initiation of testis development *(92,93)*. However, indirect evidence provided by mice lacking *FOG-2* (a GATA cofactor), which do not express *P450scc, 3β-HSD*, and *Cyp17 (94)*, suggest a role for GATA-4 and its partner FOG-2 in steroidogenic gene expression, at least in fetal Leydig cells. Additional experiments are needed to assess the role of these factors in the adult Leydig cell population.

The Class of C_2H_2 ZnF Domains

The C_2H_2 class is also known as the classical ZnF class. Dozens of C_2H_2 ZnF transcription factors, containing between 2 and 29 ZnFs, have been identified in numerous tissues (reviewed in ref. *95*). Despite the impressive numbers of C_2H_2 transcription factors, only a handful have been described in Leydig cells so far: Sp1, Sp3, gonadotropin-inducible ovarian transcription factors (GIOT)-1, and GIOT-2. Sp1 and Sp3 are ubiquitously expressed transcription factors. They contain three ZnFs that are involved in the recognition of GC-rich sequences. In Leydig cells, Sp1 and Sp3 have been shown to contribute to the expression of four genes: scavenger receptor class B type I *(96)*, LH-R *(97)*, peripheral-type benzodiazepine receptor *(98)*, vascular endothelial growth factor *(99)*.

As *Sp1*$^{-/-}$ mice die *in utero* around embryonic day 11, there is currently no data on the *in vivo* role of this factor in Leydig cell gene expression *(100)*. Two other members of the C_2H_2 class are also found in Leydig cells: GIOT1 and GIOT2. GIOT1 expression is restricted to the pituitary, adrenal gland, testis, and ovary, whereas GIOT2 is ubiquitously expressed *(101)*. Expression of both GIOT1 and GIOT2 is upregulated by gonadotropins thus suggesting that these transcription factors might be involved in the hormonal regulation of Leydig cell function *(101)*. However, no target genes have been identified for these factors.

The Superclass of HTH

The HTH is a 60 amino acid DNA-binding motif most commonly known as the homeodomain or homeobox. It is found in a family of transcription factors, called homeoproteins. Homeoproteins recognize a short DNA motif containing the core sequence TAAT and the nucleotides flanking this core are known to direct binding specificity of the various homeoproteins *(102,103)*. Homeoproteins act as transcriptional activators or repressors and the best known are the Hox factors that control body segmentation and organ development along the anterior–posterior axis. Several hundred homeobox genes have been isolated so far; some are grouped in the genome (the Hox complex) but the majority are scattered. Homeobox genes act as master switches in developmental processes of several species ranging from flies to humans (reviewed in ref. *104*). Although their implications in organogenesis, cell differentiation, and gene expression have been well-characterized in several tissues, very little is known about their expression and roles in Leydig cell differentiation and function. So far, only four homeoproteins have been reported to be expressed in Leydig cells or to have an impact on Leydig cell development: ARX, LHX9, PBX1, and RHOX4.

ARX is X-linked and expressed in the forebrain, floor plate, and testis of mouse embryos. Although, ARX is barely detectable in fetal Leydig cells, these cells fail to develop in *Arx*$^{-/y}$ mice *(105)*. The importance of ARX for the development of the fetal population of Leydig cells is further supported by mutations in the human *ARX* gene that are responsible for a syndrome called X-linked lissencephaly with abnormal genitalia *(105–107)*. No data is currently available regarding the role of ARX in the adult population of Leydig cells since *Arx*$^{-/y}$ mice die at birth as a result of severe brain defects *(105)*.

LHX9 (lim-homeobox 9) is a transcription factor expressed in testicular interstitial cells during embryonic development and its expression decreases progressively, becoming undetectable after birth *(108)*. *Lhx9*$^{-/-}$ mice

have no gonads and male mice are phenotypically female *(109)*. This is strikingly reminiscent of the SF-1-null mouse *(49,50)* suggesting that SF-1 and LHX9 might be linked in the regulation of gonadal development and male sex differentiation. Consistent with this, *SF1* expression was drastically reduced in *Lhx9*$^{-/-}$ mice *(109)* indicating that LHX9 would function upstream of SF-1 in a cascade of molecular regulators involved in gonadogenesis.

Mammalian *Pbx* (pre-B-cell leukemia transcription factor) genes (Pbx1–3) encode a subfamily family of homeoproteins that act as transcriptional regulators in numerous cell types *(110)*. As for *Arx* and *Lhx9, Pbx1* is expressed in interstitial cells during testicular development *(111,112)*. In *Pbx1*$^{-/-}$ mice, gonads form but display only rudimentary sexual differentiation *(112)*. In the testis of *Pbx1*-null mice, *SF1* expression is decreased to minimal levels and expression of *P450scc,* a marker of fetal Leydig cells and a target of SF-1, is detectable in only a few Leydig cells. Altogether, these data suggest that PBX1 is essential for gonadal development and fetal Leydig differentiation. It is unclear, however, if *P450scc* is a direct target of PBX1 or if the decrease in *P450scc* expression is the result of reduced *SF1* expression in *Pbx1*$^{-/-}$ animals.

Rhox4 is a member of a recently identified family of 12 homeobox genes that are clustered on the X chromosome and expressed almost exclusively in reproductive tissues (Reproductive hox [Rhox]) *(113)*. Of the 12 Rhox genes, only *Rhox4* is predominantly expressed in Leydig cells of adult animals. Targeted inactivation of *Rhox4* has not yet been reported and no target genes have been identified for this factor.

The Superclass of β-Scaffold

The β-scaffold domain is a globular structure that binds to the minor groove of DNA. Two transcription factors belonging to this superclass are expressed in Leydig cells where they were shown to regulate important physiological processes: NF-κB and STAT5. NF-κB is expressed in most cell types where its activity is regulated by a variety of signals including cytokines *(114)*. NF-κB regulates gene expression by binding to the GGGRNNYYCC motif found in the promoter or enhancer regions of target genes. Leydig cell are particularly sensitive to cytokines produced by testicular macrophages (reviewed in ref. *115*). In response to immune challenge or chronic inflammatory disease, these macrophages produce cytokines, such as TNF-α and IL-1, which are known to inhibit Leydig cell steroidogenesis. Recently, Lee and colleagues have reported that in response to proinflammatory cytokines, NF-κB physically interacts with the nuclear receptors Nur77 and SF-1 leading to an inhibition of their transactivation properties and ultimately a decrease in steroidogenic gene expression and testosterone production *(69)*. Importantly, this mechanism provides the first insight into the molecular regulation of Leydig cell function by macrophages.

The STAT family of transcription factors is made up of six members, STAT1–6, and they are well-known for their roles as mediators of cytokine and growth factor signaling (reviewed in ref. *116*). They activate transcription by binding as dimers to the consensus sequence TTCNNNGAA (reviewed in ref. *117*). STAT5 is the only member of this family expressed in Leydig cell where it was shown to mediate the effects of erythropoietin, growth hormone, and prolactin, which are all growth factors important for Leydig cell development and function *(118,119)*. So far, only one gene involved in testosterone synthesis, the human *HSD3B2,* was shown to be regulated by STAT5 *(120)*.

CONCLUSION AND PERSPECTIVES

The process of testis formation and Leydig cell development and function, as for all pathways of organogenesis, relies on a transcriptional network encoded in the DNA. This process requires the combinatorial action of several mediators such as signaling molecules, receptors, and transcription factors. As summarized in the present chapter, several transcription factors belonging to diverse families have been implicated in testis formation and Leydig cell development and function. Most of the transcription factors currently known to be expressed in Leydig cells have been identified through analyses of the regulatory regions of genes expressed in these cells, and especially those genes encoding steroidogenic enzymes involved in testosterone synthesis. The advent of modern biotechnologies, such as array-based gene profiling, serial analysis of gene expression, proteomics, and *in silico* screening, will allow for the rapid identification of additional transcription factors that had not previously been reported in Leydig cells. Despite the tremendous power of these approaches, the classical method of studying gene promoters still remains the technique of choice today. Because it provides an invaluable advantage over the new technologies: a known target gene for the transcription factor that allows for detailed analyses of its role and mechanism of action in Leydig cells. However, it is still a long way from a full understanding of the molecular events that regulate the specification, differentiation, and function of Leydig cells. Traditional gene inactivation experiments of transcription factors often lead to a complete agenesis of the gonads or to embryonic-lethal phenotypes thus precluding the use of this technique for the study of a particular factor in

Leydig cell development and function. Alternatives are therefore essential. These include cell-specific gene inactivation using the Cre-Lox system as well as transgenic mice overexpressing siRNA or dominant negative factors to knockdown transcription factor expression/activity specifically in Leydig cells. To drive expression of these molecules (Cre recombinase, siRNAs, dominant negatives) in Leydig cells at different times during development, it is essential to have different Leydig-specific promoters at one's disposal. The *in vitro* and *in vivo* characterization of these promoters have begun but are still a work in progress.

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24 Androgen Receptor in Leydig Cell Function and Development

Stephen M. Eacker, PhD and Robert E. Braun, PhD

SUMMARY

Testosterone (T) is a critical regulator of sexual differentiation and spermatogenesis in mammals. The effects of T are mediated by the androgen receptor (AR), a nuclear hormone receptor transcription factor. AR is expressed in many cell types throughout the organism including the T-secreting Leydig cells (LCs). Although, its expression pattern in the fetal generation of LC is unclear, AR is expressed throughout the maturation of adult LCs. Expression of AR is required for normal adult LC development as assessed by morphology, steroid output, and expression of markers of maturation. LCs in human bearing mutations in *Ar*, lack crystals of Reinke and secrete large amount of estrogen and normal to high levels of T. In contrast, mice that have congenital mutation in *Ar* produce less T than their wild-type littermates and have small, lipid-laden LCs with reduced volume of smooth endoplasmic reticulum. Marker gene analysis show that mice lacking AR fail to express a number of genes associated with normal LC maturation. Rats mutant for *Ar* show similar defects in LC morphology and steroidogenic activity. Within the context of the wild-type testis, AR functions in an ultrashort feedback loop to repress steroidogenic gene expression. Mutations in *Ar* predispose all mammals to testicular neoplasia, including LC tumors. The mechanism of tumor induction in *Ar* mutants remains unclear. Together, the studies discussed in this chapter show that AR plays a critical role in LC function and development.

Key Words: Androgen receptor; feedback regulation; Leydig cell; Leydig cell tumor; steroidogenesis; testosterone.

INTRODUCTION

The androgen receptor (AR) is a member of the nuclear hormone receptor superfamily of transcription factors *(1)*. AR is the sole receptor for the steroid hormone testosterone (T) and its more potent metabolite 5α-(DHT) dihydrotestosterone. Upon binding its ligand, AR translocates to the nucleus where it can act either to activate or repress transcription of specific target genes. Although, AR is not required for testis specification during embryonic development, it is a critical regulator of the differentiation of secondary structures in the male reproductive tract *(2)*. FLCs of the testis produce T, which stimulates AR to specify the Wolffian-derived structure, the prostate, and the seminal vesicle, and supports development of external male structures. All major cell types of the testis are present within the testis in the absence of AR signaling, although their development is defective. In the adult testis, AR signaling continues to be important to maintain spermatogenesis and regulate steroid synthesis *(3,4)*.

In this chapter, the role of AR in the development and function of LCs will be focused. First, to understand the function of AR in the LC, the dynamics of AR expression in LCs will be discussed. Then the effect of AR mutations on the three criteria used traditionally to assess LC maturation and function: morphology, steroid output, and expression of marker genes will be described. Finally, it will end with a brief discussion of the role of *Ar* mutation in LC neoplasia.

EXPRESSION PATTERN OF AR IN LCs

The availability of AR cDNAs and a variety of AR antibodies has allowed extensive characterization of AR localization, particularly in the adult testis. In this chapter, AR expression in human, rat, and mouse LCs

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have been chosen to focus. In general, the localization pattern of AR in these organisms holds true in other members of *Mammalia* when it has been investigated. Additionally, LC function is the best characterized in these species, making consideration of AR localization in these species most pertinent. As discussed extensively in other chapters in this volume, there are two types of LCs, which populate the testis during the lifetime of mammals: fetal LCs (FLCs) and adult LCs (ALCs). The embryonic origin of the FLC population remains unclear, although some evidence supports a shared origin with adrenal cells, which migrate from the mesonephros at embryonic day (e) 11.5 *(5,6)*. ALCs in rodents are derived from mesenchymal stem cells *(7)*, which differentiate from vascular smooth muscles cells and pericytes *(8)*. Although, FLCs persist into the postnatal period, ALCs predominate the interstitial cell population in the adult. In the following section, the term "interstitial cells" is used to describe any cell within the interstitium of the testis, which has not been clearly identified as a LC by either morphology or marker expression.

AR in FLCs

In the rat, FLCs are present in the embryonic gonad shortly after sex determination and begin producing T by 15 d postcoitum (dpc) *(9)*. Around the time that T production begins within the testis, AR is found within the mesenchymal cells surrounding the Wolffian ducts in the mesonephros, but is absent from the testis proper *(10)*. Starting at 17 dpc, AR is present within the interstitial cells of the testis. The identity of the interstitial cells, which express AR during this period in development is unclear. By 19 dpc there is a high level of AR immunoreactivity in the interstitium and peritubular myoid cells. This pattern of staining remains consistent through birth. Costaining of 19.5 dpc rat testes with antibodies against AR and 3β-HSD clearly demonstrates that the steroidogenic cells of the testis do not express AR at this time. The authors of this study suggest that the AR-positive cells in the interstitium could represent LC progenitors as androgens promote LC maturation in the adult (discussed next) *(10)*. The FLCs persist in the postnatal testis, though their exact fate is unclear. On postnatal day (p)5, a subset of interstitial cells stains prominently for AR; it is not clear whether these are FLCs that have acquired AR expression during postnatal development, progenitors of the ALCs, or a third undefined cell of the interstitium.

A small number of studies have reported localization of AR in the developing mouse gonad. AR messenger RNA (mRNA) is present in the interstitium as early as e13.5, demonstrated by *in situ* hybridization with a fragment of the mouse AR cDNA *(11)*. However, the one study examining immunolocalization of AR in the mouse embryonic gonad showed no evidence of expression in the interstitial cells at e14, 16, or 18 *(12)*. By p8, there is extensive expression of AR in the interstitial cells *(13)*. The identity of these cells is unclear: both FLCs and PLCs are present in the testis at this time. However, careful evaluation of cell proliferation in the postnatal testis indicated that ALCs do not begin to extensively proliferate until p11 *(14)*. Therefore, at p8, the majority of the cells in the interstitium are likely to be derived from the fetal population of LCs.

At 7 and 8 wk gestation in the human, the gonad is poorly organized and no cells within the testis express AR *(15,16)*. By 9 wk gestation, AR expression becomes apparent within the gonad, primarily staining the rat testis. Staining in the interstitial cells encompassing the nascent seminiferous cords begins on week 10 of gestation. Surprisingly, AR expression is absent from week 11 embryonic testes *(16)*. Loss of AR expression in the embryonic testis at this stage might be indicative of the regression of the first wave of LCs, which populate the fetal gonad. Alternatively, the small sample size represented in the study might mask variability in timing of the onset of AR expression. AR immunoreactivity is once again observed in the fetal gonad by 12 wk gestation when CYP11A1 positive FLCs are clearly present within a now well-defined interstitium. In contrast to the rat, a subset of CYP11A1-positive FLCs are also positive for AR expression *(15)*. The intensity of AR staining continues to increase in interstitial cells at 16 and 22 wk gestation. Although, it has been shown that the fetal population of LCs regresses during late gestation in human, by 22 wk CYP11A1 cells remain in the interstitium, staining with an albeit lower intensity.

AR Expression in ALCs

The rat's adult population of LCs begins to differentiate around p14, forming precursor LCs (PLCs) *(17)*. Extensive analyses show that by p21, PLCs express AR mRNA by quantitative *in situ* on testis sections and semiquantitative reverse transcriptase-polymerase chain reaction performed on purified PLCs *(18)*. As the PLCs develop into immature LCs (ILCs), they acquire a 30–50% increase of both AR mRNA and protein *(18,19)*. AR levels then decline significantly as the ILCs form the mature ALC population. Levels of both

AR mRNA and protein are lower in ALCs than in either PLCs isolated from p21 or ILCs from p35 testes.

Hormonal regulation of *Ar* has also been investigated in developing rat LCs. Isolated LCs from p14 rats, which were treated with the GnRH antagonist NalGlu for 1 wk resulted in a subtle decrease in AR mRNA *(20)*. If in addition to NalGlu, the animals were given exogenous LH, T, or both before LC isolation, there was a significant upregulation of AR mRNA. These data suggest that T upregulates AR mRNA expression either directly at the transcriptional level or indirectly by promoting the precocious maturation of PLCs into ILCs. Similar experiments were performed on rats treated with NalGlu on p21, 35, and 90, but instead measuring the relative staining intensity (RSI) of AR by immunohistochemical methods *(21)*. In the case of PLCs on p21 and ILCs on p35, the RSI of AR was severely decreased by treatment with NalGlu and returned to normal when either T or MENT (17α-methylnortestosterone) were coadministered with the GnRH antagonist. LH was sufficient to return AR RSI to normal levels in PLCs from NalGlu-treated rats. Interestingly, LH supplementation did increase AR RSI in ILCs, but was not sufficient to return AR RSI to control levels. Treatment of fully mature ALCs with NalGlu alone or in combination with LH, T, or MENT had no effect on AR RSI. Taken together, these data suggest that LH and T most significantly affect AR expression during postnatal maturation, and AR levels in mature ALCs are refractory to hormonal perturbations.

Immunohistochemical methods of AR localization in adult mice demonstrate very similar patterns to those observed in the adult rat. Zhou et al. *(12)* saw little or no staining in LCs before p21. On one hand, this correlates well with the observation in rats that the most intense AR staining occurs in the pubertal ILCs. On the other hand, other studies have shown staining in murine LCs as early as p8 *(13)*. The high levels of AR staining in LCs at day 21 remained constant to adulthood *(12)*. In another study of AR expression in adult mouse tissue, LC staining was lower and more variable *(22)*. This is consistent with what is observed in the adult human testis (*see* next paragraph).

Expression of AR in postpubertal human testis has been the subject of a number of investigations. Using Percoll gradient centrifugation, Namiki and colleagues *(23)* isolated human LCs from testes taken from 10 men in the age group of 25–34. Isolated human LCs bind ^{3}H-R1881, a synthetic androgen, and express AR mRNA as demonstrated by Northern blot analysis using a human cDNA probe *(23)*. The earliest studies of AR immunolocalization in human tissues used a mouse monoclonal antibody made against the human AR protein *(24,25)*. Generally, these studies agree that AR expression is prominent in the adult LC. A study of testes from older (57–69 yr old) men undergoing therapeutic orchidectomy used the PG21 rabbit polyclonal antibody directed against residues 1–21 of the human AR protein. The authors of this study used limiting concentrations of antibody to assess the relative levels of AR expression in various cell types within the testis *(26)*. They concluded that LCs express low levels of AR, lower than any other testicular cell type where AR was detected. The staining in ALCs was also variable, with some not staining positive for AR. A study of biopsies from men with idiopathic infertility *(27)* found variable staining of LCs, similar to that observed by Suarez-Quian et al. *(26)*. Similar observations were made in a study of cryptorchid testes from postpubertal men who underwent prophylactic orchidectomy. In this study, despite the cryptorchidism, the LCs showed weak/variable expression of AR.

Summary of AR Expression in LCs

The AR is expressed at variable levels during the maturation of ALCs. Generally, studies from rats and mice agree that AR expression is the highest during pubertal maturation of the ALC and then decreases in the mature ALC to a weak/variable level. The low/variable expression pattern is also observed in adult human LCs. The expression pattern of AR in FLCs is far more ambiguous. AR is clearly expressed in a subset of interstitial cells during development. However, it is impossible from existing data to discern the identity of AR-expressing cells in the interstitium. Determining the expression pattern of AR within FLCs will require careful morphological and marker analysis.

EFFECTS OF *AR* MUTATIONS ON LEYDIG DEVELOPMENT

The syndrome caused by mutations in the human *Ar* has classically been termed "testicular feminization" *(28)*. The clinical definition of this syndrome is an XY individual who appears externally female with a short, blind vagina, scant pubic and axillary hair, and undescended testes. Although, not recognized immediately as the result of mutations in *AR*, an early-proposed cause of testicular feminization syndrome (TFS) was androgen insensitivity *(29)*. Today, TFS is more correctly identified as Androgen Insensitivity Syndrome (AIS; OMIM: no. 300068), and is known to be caused by a variety of inactivating mutations in *AR*. To date, more than 600 mutations in *AR* have been identified

(30; http://www.mcgill.ca/androgendb/). Variations in the severity of these *AR* mutations lead to phenotypic variation in the presentation of AIS. More severe inactivating mutations lead to complete AIS (cAIS) with all the characteristics of TFS described earlier. A broad range of partial loss-of-function mutations leads to partial AIS (pAIS), often phenotypically characterized by intersex external genitalia. The milder forms of AIS are encompassed by Reifenstein's syndrome (OMIM: no. 312300), which is often used to describe patients with androgen resistance accompanied by microphallus, hypospadia, and undescended or partially descended testis, but general male habitus.

The classic rodent models of AIS are the Stanley–Gumbreck *Tfm* rat and the *Tfm/y* mouse (31,32). Both of these models recapitulate many of the human AIS phenotypes, including pseudohermaphrodism, undescended testes, and androgen resistance. The *Tfm/y* mouse represents a null allele of *Ar* caused by a single base deletion resulting in a frameshift mutation (33). The *Ar* mutation in the Stanley–Gumbreck *Tfm* rat is caused by a point mutation, which causes a substitution of a conserved residue in the steroid-binding domain (34). This substitution results in a full-length protein, which has severely diminished, but still present, androgen-binding activity. In this way the *Tfm* rat is perhaps more accurately characterized as a model of pAIS.

In this section, how these *Ar* mutations affect LC function and development will be discussed. Three measures of LC maturation in *Ar* mutants: morphological development, steroidogenic output, and marker gene analysis will be focused.

Effects of AR *Mutations on LC Morphology*

The morphological changes that LCs undergo during development are extensively discussed elsewhere in this volume. The goal of this section is to detail how mutations in the *Ar* affect the stereotyped development of the LC at the histological and ultrastructural levels. Oftentimes, histological observations have led researchers to conclude that *Ar* mutations cause hyperplasia of the LC. However, in mouse and human *Ar* mutants, systematic estimation of LCs has revealed that LC numbers are the same or slightly decreased compared with normal controls (35,36). The illusion of hyperplasia is because of a decrease in seminiferous tubule diameter, which results from germ cell arrest. In human with cAIS, proliferation of spindle-shaped cells in foci or in large regions of the testis is often observed (37–39). Morphologically, these cells appear similar to ovarian stromal cells. Some researchers identify these simply as stromal cells, leaving open the possibility that these spindle-shaped cells are in fact undifferentiated progenitor LCs (39). As will be discussed next, T has a positive effect on the maturation of rat LCs in culture (40). A similar role for AR signaling in human LCs seems likely. The appearance of stromal cells in the interstitium has also been described in a number of cases of patients with pAIS. Although, both pAIS and cAIS patients in these cases had cryptorchid testes, it is likely that the presence of stromal cells is a consequence of the absence of AR because stromal cell proliferation is not observed in cases of cryptorchidism in the absence of AIS (39).

A large increase in cytoplasmic volume is one of the most obvious changes that occur during LC maturation. The progenitors of the mature adult LC are small, spindle-shaped fibroblasts that populate the interstitium (41). Over the course of pubertal development, the small spindle cells develop into the large, polygonal LC. The dominant feature of the voluminous cytoplasm of LCs is the smooth endoplasmic reticulum (SER). The SER is the site of steroid biosynthesis after the conversion of cholesterol to pregnenolone in the mitochondria. Accordingly with this function, proliferation of SER accompanies the rise in steroid output during puberty. In the *Tfm/y* mouse, there is a noticeably smaller amount of SER in the cytoplasm of LCs (42–44). SER in murine LCs often forms striking concentric whorls, a feature rarely observed in other species (Fig. 1). Normally, whorls of SER form during early puberty and are considered indicative of mature SER in LCs of the mouse. The whorl configuration of SER is notably absent from *Tfm/y* LCs (42,43).

In contrast with observations in the *Tfm/y* mouse, human with cAIS or pAIS have seemingly normal amount of SER (38,45–47). However, patients with AIS lack a human-specific morphological feature of mature LCs: crystals of Reinke. These crystalloid structures of unknown composition and function are formed in human LCs around the time of puberty. They are found in nearly every adult human LC. Virtually, in all reported cases of cAIS, no crystals of Reinke have been observed, and they have only rarely been observed in cases of pAIS (37,38,39,45,47). It has been suggested that LCs in AIS patients are of fetal origin or in the least fetal-like, as a result of lack of crystals of Reinke even at the height of steroid production in human FLCs (38,45). The significance of this unique structure's absence is unclear. The appearance of the crystals of Reinke at puberty has led some to speculate that they may be involved in steroidogenesis. However, steroid levels in AIS patients tend to be

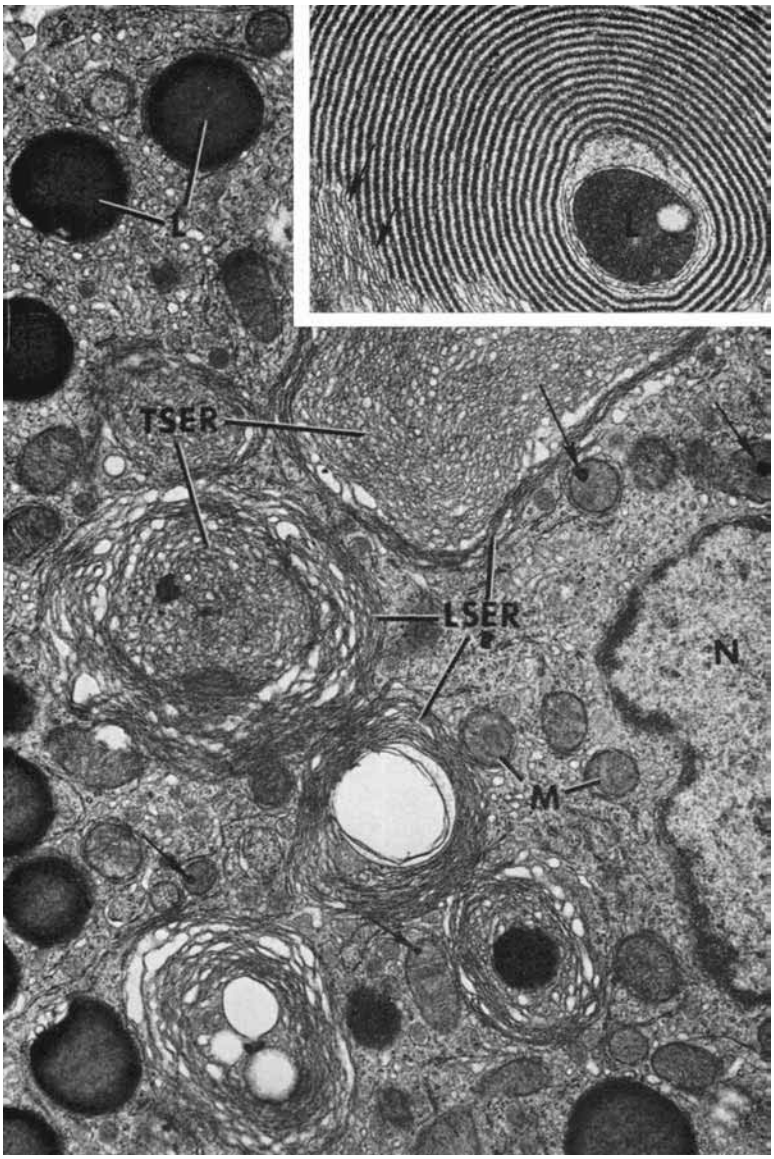


Fig. 1. A transmission electron micrograph of a Leydig cell from a wild-type mouse testis. The adult Leydig cell is characterized by abundant tubulovesicular SER and lamellar SER. Note close association of osmiumophilic lipid droplets (L) with SER. Also, associated with the SER are the mitochodria (M), the site of side-chain cleavage of cholesterol into pregnenolone. The nucleus (N) demonstrates the typically diffuse pattern of chromatin observed in Leydig cells. The inset panel displays the whorl configuration of SER commonly observed in the murine Leydig cell, which, in this case, is associated with a lipid droplet. Magnification ×15,500, inset ×25,500. (Image reprinted from *[42]*, courtesy of *Biology of Reproduction*.)

normal or higher than normal, indicating that steroidogenesis is not defective in the absence of crystals. Additionally, crystals of Reinke are observed in the LCs of cryptorchid individuals, suggesting that their absence in AIS patients is not attributable to failure of testicular descent *(48)*.

Lipid droplets are also a prominent feature of maturing LCs. In mice, large quantities of lipid droplets are found in the cytoplasm of LCs starting at puberty. In the rat, the appearance of lipid droplets is associated with the transition from PLCs to ILCs. Although, the progenitor and immature stages have not been precisely defined in other species, increase in the number of lipid droplets is associated with conversion of LCs from a spindle-shaped fibroblast to a larger, polyhedral cell. The number and size of lipid droplets peaks during puberty and subsequently declines in adulthood. Lipid droplets are depleted from immature LCs when they are chronically stimulated with human chorionic gonadotrophin (hCG), suggesting that lipid droplets are depleted as LH-stimulated steroidogenic output increases into adulthood.

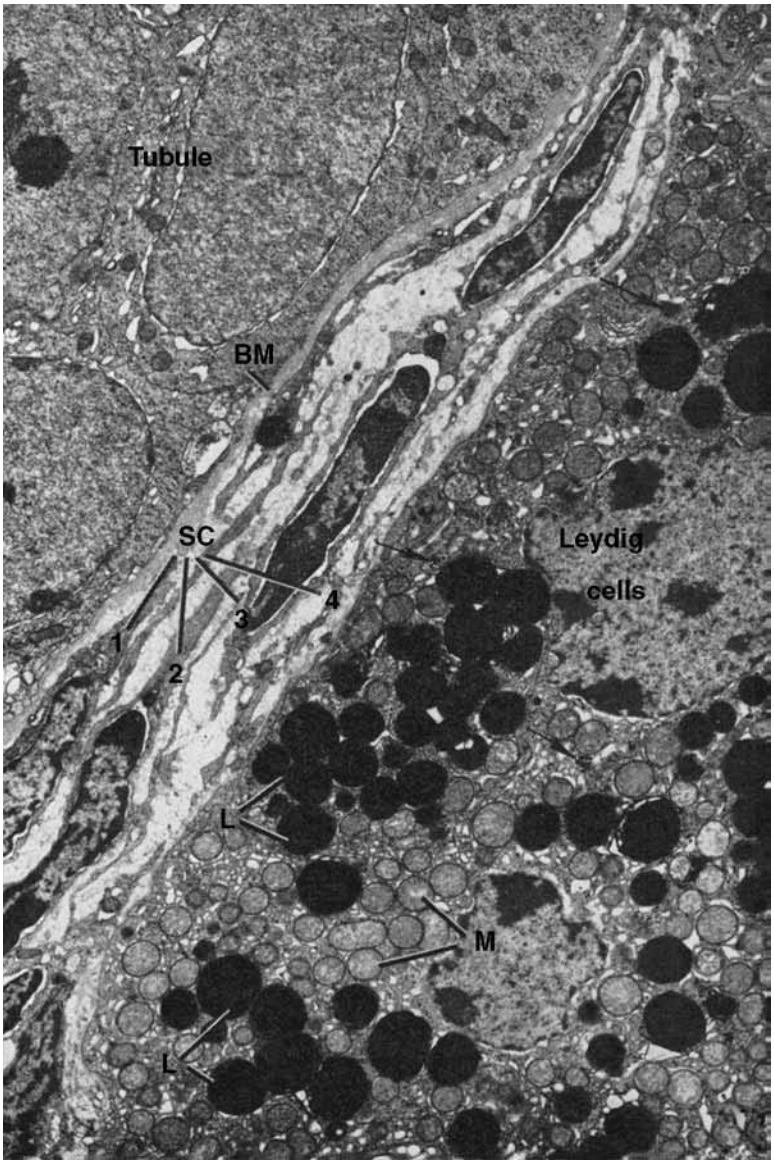


Fig. 2. A transmission electron micrograph of a Leydig cell in close association with the seminiferous tubule in the testis of a *Tfm/y* mouse. Note the relative absence of SER compared with the wild-type Leydig cell (Fig. 1) and the increased density of lipid droplets (L) and mitochodria (M). Separating the Leydig cell from the tubule are the basement membrane (BM) and an unusual, multilayered group of peritubular myoid cells, also termed sheath cells (SC). Magnification ×7500. (Image reprinted from ref. *42*, courtesy of *Biology of Reproduction*.)

LCs from *Tfm/y* mice contain numerous large lipid droplets that are retained into adulthood *(42,43;* Fig. 2). Chung and Hamilton *(43)* analyzed the lipid content of *Tfm/y* testis, measuring total lipids, phospholipids, and both esterified and free cholesterol. They compared lipid levels in *Tfm/*y testis with wild-type testes, and wild-type testes rendered surgically cryptorchid. In both cryptorchid and *Tfm/y* mice there was a significant increase in all classes of lipids measured. The most significant increase was in esterified cholesterol, which increased 10-fold over wild-type controls. At first glance, these data suggest that cryptorchidism in *Tfm/y* mice is the cause of the accumulation of lipid droplets in LC cytoplasm. However, the authors of this study demonstrate that there is a more extensive accumulation of lipid in the Sertoli cells of surgically cryptorchid mice, whereas in *Tfm/y* testis, the accumulation of lipid is most dramatic in the interstitial compartment. The difference in lipid distribution between cryptorchid and *Tfm/y* testes might reflect the differences in the developmental history of these two models. The wild-type mice were postpubertally rendered cryptorchid and

Table 1
Relative Serum Hormone Levels in *AR* Mutants

Mutation	*Serum LH*	*Serum T*	*Serum A*	*Serum E_2*	*References*
Homo sapiens					
cAIS (adult)	Normal-high	Normal-high	n.d.	High	*56–58*
cAIS (neonatal)	Low	Low	n.d.	n.d.	*61*
pAIS (adult)	High	Normal-high	High	High	*62,130*
pAIS (neonatal)	Normal-high	Normal-high	n.d.	n.d.	*61*
Reifenstein's syndrome (adult)	High	Normal	Normal	Normal-high	*62,131*
R. norvegicus					
Stanley–Gumbreck *Tfm* (adult)	High	Normal-high	High	High	*74,75,77*
Stanley–Gumbreck *Tfm* (neonatal)	n.d.	Normal	n.d.	n.d.	*75*
M. musculus					
Tfm/y (adult)	High	Low	n.d.	n.d.	*79,80*
Tfm/y (neonatal)	Normal	n.d.	n.d.	n.d.	*81*
$Ar^{invflox(ex1\text{-}neo)/Y}$	High	High	n.d.	Normal	*84*
$Ar^{flox/Y}$; *Amh-Cre* (*SCARKO*)	Normal	Normal	n.d.	n.d.	*85*
$Ar^{flox/Y}$; *Amh-Cre* (*S-$Ar^{-/Y}$*)	High	Low	n.d.	n.d.	*86*

therefore experienced normal pubertal development of the testis unlike their *Tfm/y* counterparts. In contrast to the *Tfm/y* mouse, the LCs of humans with AIS have relatively few lipid droplets. This may reflect differences in steroidogenic output between human and mice that lack AR. On the other hand, human have smaller stores of lipid in their LCs, making the significance of this species-specific difference unclear.

Lipid metabolism and synthesis are also likely affected by a paucity of peroxisomes in *Tfm/y* mice. Reddy and Ohno *(44)* investigated the distribution of peroxisomes in the LCs of *Tfm/y* mice using diaminobenzidine to localize endogenous peroxisomal catalase activity. Peroxisomes appear as dark precipitates within membrane-bound structures and show a close association with steroidogenic SER in wild-type testis. However, in adult *Tfm/y* testis, there were very few peroxisomes and peroxisomes that were present stained with a lower intensity. The precise role of peroxisomes in steroidogenic cells is unclear: they have been implicated in both mobilization of free cholesterol for steroidogenesis *(49,50)* and the *de novo* synthesis of cholesterol *(51)*.

Effects of Ar *Mutations on Steroidogenic Output*

Steroidogenic output is the critical measure of LC function. Over thes course of LC maturation, not only does LH-stimulated steroid output increase, but the composition of secreted steroids also changes *(52)*. The focus of this section will be the role of T, signaling through the AR, in promoting the maturation of LC steroidogenesis. There are considerable differences in the steroidogenic output among human, rat, and mouse *Ar* mutants (Table 1). Certainly, some of the differences are attributable to species-specific differences in HPG axis physiology and development. But considerable differences in LC steroid output are also because of within-species variation. In humans, there is a high deal of variability in phenotypic manifestations of AIS, including variation in steroid output, due in part to the severity of the mutation in *AR*. Genetic variation in modifiers of AIS phenotypes has also been observed even within families that share a common mutation in *AR (53)*. This concept is also well illustrated by differences in steroid output as a result of genetic heterogeneity between inbred strains of mice *(54,55)*. Generally, results from studies of rodent *AR* mutants are more consistent between studies, probably because of the controlled genetic background of the animals. With these differences in mind, the effects of *Ar* mutations on steroidogensis for each species will be discussed individually (Fig. 3).

Steroid Output in Cases of Androgen Insensitivity Syndrome

The diagnosis of AIS often involves the measurement of serum hormone levels. These analyses reveal that, in most cases, LCs are functional, producing a large amount of T under the regulation of LH *(56)*. In fact, in most cases of cAIS, patients have above normal levels of both LH and T for normal men *(57,58)*. High levels of LH are believed to be the result of absence of negative feedback regulation at the hypothalamic–pituitary level. In infants between ages 1 and 6 mo, there is a well-documented

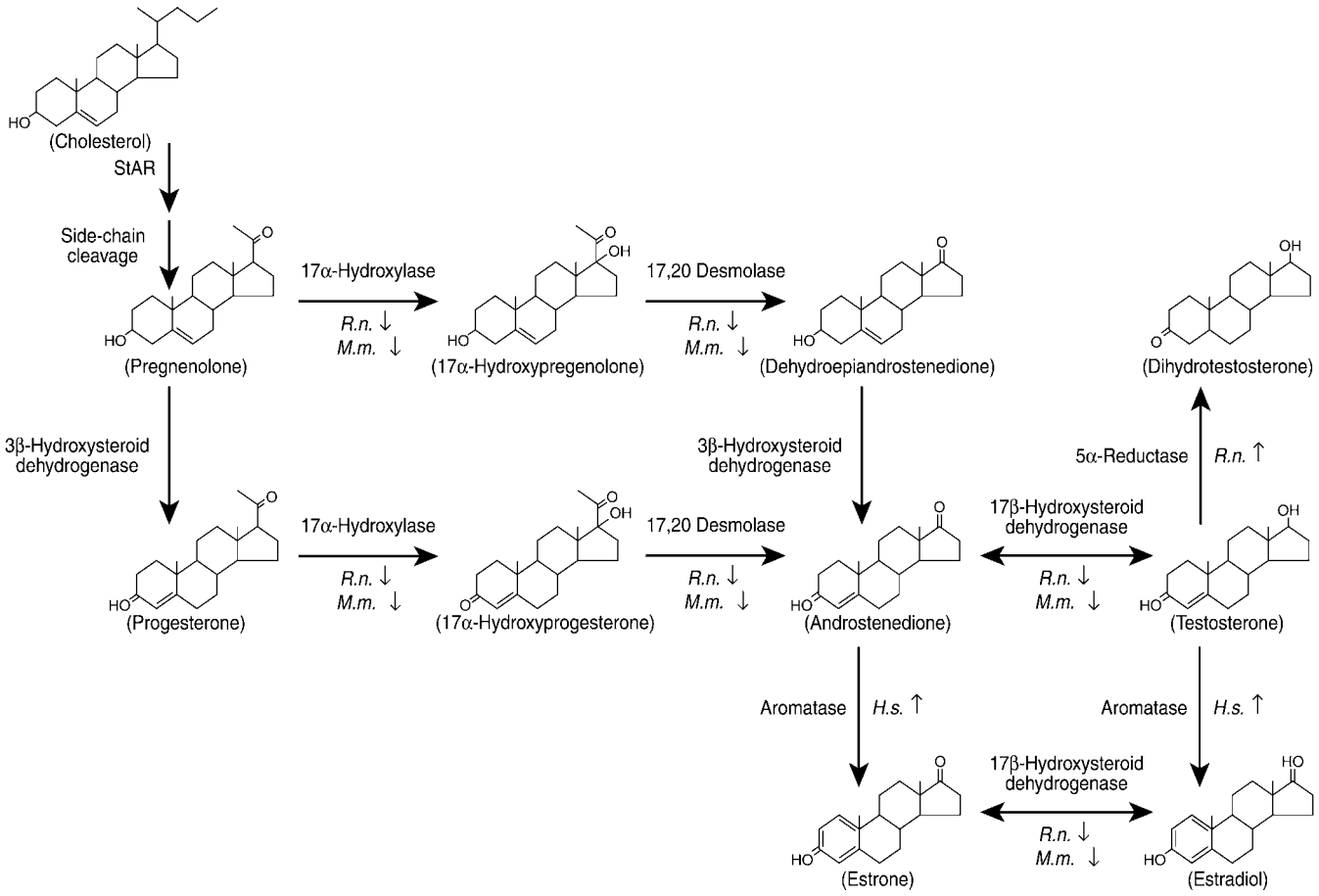


Fig. 3. A summary of changes in steroidogenic activities in Leydig cells from *AR* mutants. Arrows indicate changes in individual steroidogenic activities in *Homo sapiens* (*H.s.*), *Ratus norvegicus* (*R.n.*), and *Mus musculus* (*M.m.*) associated with mutation in *Ar*. *See* text for details and references.

phenomenon of a transient postnatal rise in serum T *(59)*. This is believed to be caused by residual hCG from the mother. Existence of this phenomenon itself indicates that at the time of birth there are LH-responsive LCs present within the testis. In a meta-analysis of 58 verified cases of cAIS and pAIS, there was no demonstrable defect in serum T levels during the postnatal period *(60)*. A different result was obtained in a longitudinal study of 10 cAIS and five pAIS patients from day 2 to 7 until 90 d postpartum *(61)*. In this study, serum T rose in pAIS patients from 2 to 7 d until day 90, the time of normal T peak during the postnatal period. In contrast, patients with cAIS showed only a slight increase in serum T during this period. This difference in T secretion is probably not because of a defect in LC function. Serum LH was significantly higher in the pAIS group than in the cAIS group, which is the most likely cause of differences in T secretion. After administration of exogenous hCG, both cAIS and pAIS patients produced a significant increase in serum T, which was statistically indistinguishable between the groups. Other studies confirm hCG responsiveness of LCs in cases of pAIS and cAIS in infants *(60)*. When treated with GnRH, both groups had elevated levels of serum LH, but the effect was significantly higher in the pAIS group than in the cAIS group. This variation might reflect a developmental difference between pAIS and cAIS at the level of the pituitary.

Studies analyzing serum hormone level in adults with *Ar* mutations have generally shown that there is little or no defect in T synthesis in LCs. Generally, serum levels of T are in the normal to slightly above normal range, consistent with the higher levels of serum LH observed *(56,62–66)*. Whereas serum T levels are in the high-normal range, levels of serum estrogens in AIS patients are dramatically higher than in normal postpubertal men. High levels of serum estrogens are observed in all varieties of AIS from Reifenstein's syndrome to cAIS *(58,62,65,66)*. The source of serum estrogens has become the focus of a number of investigations. One approach is to measure the concentration of estrogens in the spermatic vein exiting the testis and compare it with its concentration in peripheral blood.

Generally, these studies agree that the testis is the primary source of estradiol (E_2) in the bloodstream *(65,66)*. Although, estrogen levels rapidly decrease after gonadectomy, this does not rule out the possibility that androstenedione (A) and T are being aromatized to estrone (E_1) and E_2 in the periphery. Fibroblasts cultured from prepubertal pAIS patients have significantly elevated levels of aromatase activity compared with normal prepubertal controls *(67)*. Despite this difference, it is clear from experiments using radiolabeled steroids that the majority of E_2 is synthesized in the testis *(65,68)*. These experiments also demonstrated that the majority of E_1 is produced as a result of peripheral metabolism of A and T, as well as E_2 conversion into E_1. These results are supported by experiments where testicular slices or homogenates from AIS testes are incubated with radiolabeled steroids *(68,69)*. In these experiments, both C_{21} and C_{19} steroids can be converted to significant amounts of E_1 and E_2. However, most E_1 is converted rapidly to E_2 in agreement with the relative concentration of E_1 in peripheral vs spermatic vein serum *(69)*. Despite the wealth of evidence of enhanced aromatase activity in the AIS testis, there is no clear evidence for an increase in LC aromatase expression. The mechanism of elevated aromatase activity in the AIS testis remains unresolved.

Adults with AIS respond to hCG stimulus with increased steroid output *(57,60,68,70)*. Treatment of a 17-yr-old AIS patient for 4 d with hCG resulted in a 50% increase in serum T and a 100% increase in urinary estrogen *(70)*. A milder effect was also observed by French and colleagues *(68)* when treating a 25-yr-old AIS patient with hCG: they observed only a modest increase in urinary 17-ketosteroids. Balducci et al. *(57)* demonstrated in two individuals with AIS that a single dose of hCG caused an increase in steroid production. Curiously, one patient in this study responded with an increase in T synthesis whereas the other patient showed a sharp spike in E_2 synthesis at 24 h post-treatment. The significance of these differences is unclear. However, these studies do make it clear that human with AIS have LCs with functional LH receptors, which are capable of stimulating steroidogenesis.

Steroidogenesis in the Stanley–Gumbreck Tfm Rat

Like human with AIS, Tfm rats exhibit high levels of serum LH because of a lack of negative feedback control on the pituitary *(71–73)*. There is disagreement in the literature concerning the levels of T produced by the Tfm rat. In an early study, analyses using the double-isotope derivative method demonstrated that serum T levels were approx 25% of normal *(74)*. In later studies, serum T measured by chromatographic techniques *(72)*, or now-standard radioimmunoassay procedures *(73,75)*, show that the Tfm rat produces a large amount of T. In the case of Purvis et al. *(72)* and Chung and Allison *(75)*, the Tfm rat produced more than twice as much T as wild-type controls. It is unclear whether the differences in serum T levels observed by these investigators are the result of differences in assay protocol, sampling error, age of animals used in the study, or other real biological phenomena.

Analysis of steroid metabolism has provided a consistent picture of biochemical defects in the LCs of Tfm rats. Like studies in human, many of these analyses rely on incubation of testis slices or homogenates with radiolabeled steroid intermediates to determine specific activities. Incubation of ^{14}C-pregnenolone with testis slices of Tfm rats and normal littermates revealed a defect in 17β-HSD activity in the Tfm testis *(76)*. This was manifested by a 2–15-fold increase in accumulation of A in Tfm testis incubations compared with wild-type. Accordingly, there was a 2–20-fold enrichment of T in wild-type testis slices compared with Tfm. In this same study, a reduction in the ratio of progesterone to 17-(OHP) hydroxyprogesterone indicated a modest decrease in 17α-hydroxylase activity. However, unlike human with AIS, very little E_1 or E_2 was produced from pregnenolone by Tfm testis slices; there was only a 1.3-fold increase in estrogen production compared with wild-type. The deficiency in 17β-HSD activity was also confirmed by incubation of ^{3}H-progesterone with Tfm testicular homogenates *(77)*. In this study, the authors demonstrated accumulation of labeled androstanediol and androsterone in adult Tfm rat slices whereas in wild-type adult slices, T was the major product. These results are consistent with the absence of normal LC maturation because of prepubertal LC primarily produce 5α-reduced steroids. The findings of reduced 17α-hydroxylase and 17β-HSD activities and enhanced 5α-reductase activity in Tfm rats is supported by several later studies *(52,72,73)*.

That the developmental defect in acquiring the ability to synthesize T could be the result of an absence of AR-signaling, was demonstrated using isolated rat precursor LCs in culture *(40)*. Three-day treatment of cultured PLCs with LH only modestly increased the amount of T secreted into the media. However, when PLCs were provided both LH and DHT together, there was a highly significant increase in T production. Hardy and colleagues interpreted these results to suggest that AR is required developmentally to promote

the maturation of PLCs into ILCs that secrete large amount of T. This is consistent with the developmental defects observed in steroid synthesis in the Tfm rat. The development of Tfm LCs is probably also hindered by a reduced number of LH receptors *(78)*. FLC production of steroids is unaffected in the Tfm rat, suggesting that developmental defects in steroid output are restricted to the adult generation of LCs *(75)*.

Steroidogenic Output in the *Tfm/Y* Mouse

Serum T levels in *Tfm/y* mice are severely decreased despite the very high levels of LH *(79,80)*. As observed in the Tfm rat, there is no defect in synthesis of T in the neonatal testis, again, suggesting that AR is required only in ALCs for acquisition of normal steroidogenesis *(42)*. In the adult *Tfm/y* testis, as in the Tfm rat, there is a severe defect in the conversion of A to T as measured by conversion of ^{3}H-androstenedione in testis minces *(42)*. Again, this is indicative of defective 17β-HSD activity. However, when ^{3}H-pregnenolone is used as a substrate in testicular homogenates from *Tfm/y* mice, the majority of the precursor is converted into C_{19} steroids such as progesterone and allopregnenolone, consistent with a defect in 17α-hydroxylase activity *(80)*. Murphy and Shaughnessy *(80)* assessed each individual postside-chain cleavage activity in the androgen synthetic pathway. They found a 41-fold decrease in 17α-hydroxylase activity and an astounding 1000-fold decrease in 17β-HSD activity. A portion of the loss of both of these activities could be ascribed to cryptorchidism as wild-type mice rendered cryptorchid, showed a significant decrease in both activities. However, the vast majority of the decrease in steroidogenic activity is because of the absence of AR. The differences in enzyme activity between wild-type and *Tfm/y* begin during puberty, around p30 *(81)*. This could reflect the same phenomenon observed by Hardy et al. *(40)* where LH and DHT together supported pubertal maturation of PLCs to steroidogenically active ILCs.

Correlations between steroidogenic activities and mRNA levels in *Tfm/y* mice have been drawn out by a number of studies. Reduction of 17α-hydroxylase activity observed in the *Tfm/y* adult is mirrored by a reduction in *Cyp17a1* mRNA in whole testis *(81)* or in isolated LCs *(82)*. The mRNA coding for 17β-HSD activity in the mouse testis, *Hsd17b3*, is virtually absent from *Tfm/y* LCs. Interestingly, the mRNA encoding an adult-Leydig-cell-specific 3β-HSD, *Hsd3b6,* was virtually absent from *Tfm/y* LCs. This is surprising because *Hsd3b6* represents 70% of the total *Hsd3b* transcript in the adult testis *(83)*, yet 3β-HSD activity is not diminished in *Tfm/y* testis *(81)*.

Recently, several groups have published reports of novel targeted alleles of *Ar* *(84–86)*. Holdcraft and Braun *(84)* generated a hypomorphic conditional allele using inverted loxP sites flanking exon 1 designated $Ar^{invflox(ex1\text{-}neo)}$. A neomycin resistance (neo^{R}) cassette placed in the first intron contains a cryptic splice acceptor, resulting in stochastic splicing of *Ar* exon 1 within neo^{R}. The stochastic splicing results in a truncated message, reducing overall *Ar* transcript and protein. In the absence of any expression of *Cre*, $Ar^{invflox(ex1\text{-}neo)/Y}$ animals have normally descended testes, mild hypospadia, a 25-fold increase in serum LH, and a 40-fold increase in serum T. Clearly, steroid production is not diminished as in *Tfm/Y* animals, yet the high levels of LH indicate a defect in negative feedback regulation at the level of the pituitary. It is not clear if the amount of AR in $Ar^{invflox(ex1\text{-}neo)/Y}$ is sufficient to promote LC maturation, or if normal testicular descent is the key to the high T output. In many ways, these animals may prove to be a good model for Reifenstein's syndrome. In contrast to the phenotype of $Ar^{invflox(ex1\text{-}neo)/Y}$, the Sertoli cell-specific *Ar* mutant ($S\text{-}Ar^{-/Y}$) generated by Chang and colleagues has normal levels of LH and reduced levels of T. These investigators suggest that the absence of a paracrine factor, perhaps Anti-Müllerian hormone (Amh), secreted from Sertoli cells causes reduced steroidogenesis.

De Gendt and colleagues *(85,87)* have characterized LC function in their Sertoli cell-specific *Ar* mutant (*SCARKO*). Unlike the other two new *Ar* mutants, *SCARKO* males have LH and T levels that are statistically indistinguishable from wild type. However, characterization of LC-specific gene expression demonstrated significantly elevated levels of *Cyp11a1*, *Hsd3b1*, and *Cyp17a1*, after correcting for LC numbers. In contrast to $S\text{-}Ar^{-/Y}$ mutants, it appears that steroidogenic gene expression is upregulated in *SCARKO* LCs. It seems likely that the upregulation of the steroidogenic genes compensates for the nearly 50% reduction of LC number in the *SCARKO* mutant. Also, implicit in De Gent et al. analysis is that *Ar* is required in Sertoli cells for maximal proliferation of LCs. The mechanism by which AR in Sertoli cells stimulates Leydig proliferation is unclear at this time.

Markers of LC Maturation in AR Mutants

Development of the adult population of LCs has been classically observed by changes in morphology and steroid output. Modern molecular biology techniques have allowed for the characterization of changes in gene expression, which accompany the well-studied progression of adult LC development. These genes are

involved in steroidogenesis as well as other functions in the adult testis, which remain unclear. How mutations in *Ar* affect the expression of these transcriptional markers of LC maturation will be discussed, with a focus on the mouse, where these changes have been best characterized *(82)*.

As described in the previous section, there are several defects in LC steroidogenesis in *Tfm/y* mice. The most striking deficiency is the absence of 17β-HSD activity. In adult mouse LCs, there are two genes that encode this activity: *Hsd17b3* and *Hsd17b7 (88)*. In human, *HSD17B3* encodes the essential isozyme for T synthesis: human with mutations in *HSD17B3* develop pseudohermaphrodism similar to AIS *(89)*. Murine LC expression of *Hsd17b3* and seven peaks in adulthood with expression of the three isozyme beginning around p25 *(82,88)*. As previously discussed, *Hsd17b3* is virtually undetectable in *Tfm/y* LCs. However, it is not known, if *Hsd17b7* expression is affected by the absence of AR in LCs. It is also not known what the relative contributions of HSD17B3 and HSD17B7 are to total 17β-HSD activity in LCs. As is the case for *Hsd17b*, there are two isozymes encoding 3β-HSD activity in murine LCs: *Hsd3b1* and *Hsd3b6*. Levels of *Hsd3b1* do not follow any clear developmental trend *(82)*, but *Hsd3b6* is expressed exclusively in ALCs *(82,83)*. Also, as previously discussed, *Hsd3b6* expression is virtually absent from the *Tfm/y* testis. Again, the relative contribution of each of these isozymes to total 3β-HSD activity is unclear. Although, cryptorchidism alone decreases expression of both *Hsd3b6* and *Hsd17b3,* the effect is not sufficient to assign the decrease to failure of testicular descent alone. Instead, it is likely that the absence of these two transcripts from ALCs is the consequence of a developmental defect caused by the absence of AR. This is also true for *Cyp17a1*, the expression of which is associated with pubertal maturation and is severely reduced in *Tfm/y* LCs *(81)*.

Genes not involved in steroidogenesis are also affected in *Tfm/y* LCs. The transcripts encoding *Insulin-like 3* (*Insl3,* also known as *RLF* and *Ley I-L*) and *Prostaglandin D2 synthase* (*Ptgds*) are downregulated in adult *Tfm/y* LCs. *Insl3* encodes an insulin-like peptide hormone, which is expressed in FLCs and is required for testicular descent *(90,91)*. Although, both *Tfm/y* and *Insl3*$^{-/-}$ mice fail in testicular descent, the phenotypes in these two mice are unrelated (reviewed in ref. *92*). During male fetal development, INSL3 promotes the regression of the cranial suspensory ligament (CSL), allowing the testis to move dorsally into the inguinal space. During postnatal development, T signaling through AR, promotes retraction of the gubernaculum, thus, completing testicular descent. Testicular descent is therefore, a biphasic process where INSL3 promotes the fetal phase and T promotes the postnatal phase *(93)*. The fetal phase of testicular descent is unaffected in *Tfm/y* mutants, but the postnatal phase is disrupted, leading to cryptorchidism. After peak expression in FLCs, *Insl3* levels rise again in the adult population of LCs, beginning in the rat at p20 and in the mouse at p15 *(94,95)*. In both species, *Insl3* expression peaks in adulthood, where it is expressed exclusively by LCs. Expression of *Insl3* in prepubertal and adult *Tfm/y* LCs is significantly reduced. The function of *Insl3* in ALCs is unclear, but it might act as a paracrine factor supporting germ cell survival *(96)*.

During testicular development, *Ptgds* is first expressed in the presumptive mouse Sertoli cells during testis specification *(97)*. The *Ptgds* message remains localized within the seminiferous tubule until p5, after which the message is undetectable until p30 *(98)*. After this time, the *Ptgds* message is found primarily in the interstitial space as measured by both real-time reverse transcriptase-polymerase chain reaction and *in situ* hybridization. In *Tfm/y* LCs, *Ptgds* does not undergo the normally observed pubertal rise in expression *(35)*. The function of PTGDS in the postnatal testis remains unclear. Some evidence suggests that PTGDS can function as a retinoid carrier *(99)*. Retinoic acid is a crucial regulator of spermatogenesis, making the possibility that Ptgds could influence retinoid transport an intriguing one *(100)*. However, targeted mutation of *Ptgds*, had no effect on male reproductive function *(101)*.

ANDROGEN-MEDIATED FEEDBACK REGULATION OF T SYNTHESIS IN LCs

It has been long recognized that T has a role in regulating its own synthesis by negative feedback regulation of LH synthesis and secretion. T also functions in an "ultrashort" feedback loop within the LC to decrease the rate of its own synthesis *(102)*. The first observation of this phenomenon was made in a study of the effects of FSH and steroid on LH receptors in the hypophysectomized rat *(103)*. In this study, the hypophysectomized rats were treated with testosterone propionate (TP), and their rate of LH-stimulated T synthesis was then measured in vitro. After 4 d of TP injection, there was a statistically significant decrease in LH-stimulated T synthesis compared with vehicle-treated controls. The change in T synthesis rate was not because of changes in LH receptor number as there was

no change in ^{125}I-hCG bound between the groups. Although this result was not the main focus of the report by Chen et al. *(103)*, the results spawned other investigations into the role of T in regulating its own synthesis within the testis.

Again, using the hypophysectomized rat model, Purvis and colleagues *(102)* showed that 5 d of treatment with DHT propionate (DHTP) diminished both Adiol and T synthesis in hCG-stimulated testes in vitro. These effects were also observed in cultures of crudely isolated LCs, whether stimulated with hCG or db cyclic adenosine monophosphate (cAMP). The authors also concluded that short-term, rather than chronic, treatment with DHTP was not sufficient to repress hCG-stimulated T synthesis; although, it should be noted that a low (7 n*M*) concentration of DHTP was used to make this conclusion. Reduction of Adiol and T synthesis by DHTP was recapitulated in primary testicular cell culture from rats treated for 2 d with the synthetic androgen R1881 *(104)*. This study also demonstrated that the antiandrogen cyproterone acetate could enhance hCG-stimulated T synthesis in culture, suggesting that the repression of T synthesis was through an AR-mediated pathway. The short feedback regulation of T synthesis is not in place in testicular cells cultured from neonatal rat testis, indicating that FLCs might not be subject to this phenomenon *(105)*. This finding is not surprising as evidence indicates that FLCs in the rat do not express AR *(10)*.

The previously described studies illustrate long-term suppression of T-synthesis, but other studies have shown that T can have more rapid effect on steroidogenesis. In vitro perfusion of adult rat testes with T caused a significant decrease in LH-stimulated T synthesis within 20 min *(106)*. This rapid suppression of T synthesis showed a linear dose–response relationship and was also reversible within 20 min after T withdrawal. Coperfusion of testes with A suppressed the T-mediated repression in T synthesis, showing that the rapid block in T synthesis occurs before 17β-HSD-mediated metabolism of A–T. Using limiting concentrations of progesterone (P) coperfused with T, it was demonstrated that the conversion of P to 17OHP was blocked by T. The nature of the rapid block in T synthesis by T appears to be by competitive inhibition of 17α-hydroxylase activity as the K_m for P was increased in the presence of T whereas the velocity of the conversion of P to 17OHP was unaffected *(107)*.

Studies of 3β-HSD activity in hypophysectomized rats showed that there is rapid T-mediated inhibition of this activity as well *(108)*. Within 2 h of injection of DHT, there was a dose-dependent inhibition of 3β-HSD activity. The mechanism of 3β-HSD inhibition is unclear; pretreatment with cycloheximide abolished DHT's inhibition of 3β-HSD activity, suggesting that the mechanism does not involve competitive inhibition.

The rapid inhibition of T-synthesis demonstrated by Darney and colleagues occurs at high, but physiologically relevant concentrations of T. This contrasts with the long-term treatment with T leading to suppression of T-synthesis observed by other investigators, where lower levels of androgens were required to observe the effect *(102,104)*. Further investigation of the long-term suppression by R1881 showed that hCG-stimulated 3β-HSD activity decreased after 3 d of treatment with the synthetic androgen *(109)*. Cyproterone acetate acted to enhance hCG-stimulated 3β-HSD activity, again suggesting an AR-mediated mechanism *(109,110)*. Unlike the rapid block in T-synthesis by competitive inhibition of CYP17A1, the long-term treatment of cells with androgens did not affect the affinity of 3β-HSD for its substrate, but instead decreased the velocity of the reaction, consistent with a decrease in total amount of enzyme present within the cell.

Consistent with a reduction in the amount of enzyme as the mechanism for repression of 3β-HSD activity, T is capable of downregulating *Hsd3b* mRNA in cultured mouse LCs *(111)*. In these experiments, addition of aminoglutethimide (AG), an inhibitor of cholesterol side-chain cleavage, was necessary to observe cAMP induction of *Hsd3b*. It is the most likely that addition of AG is required in order to block *de novo* synthesis of T, which in turn would repress *Hsd3b* transcription. In the absence of AG in the media of the cAMP-stimulated LCs, media concentration approached 1.5 μ*M*. The addition of AG limited the concentration in the media to less than 100 n*M*.

Interestingly, T-mediated repression of 3β-HSD activity is strain-dependent in mice *(112)*. As previously demonstrated in rats, 3β-HSD activity in LCs from C57BL/6 mice can be repressed by addition of DHT or enhanced by treatment with antiandrogens such as flutamide. However, 3β-HSD activity in LCs from C3H/HeJ mice is not affected by either androgens or antiandrogens. This modifier androgen responsiveness is dominant because LCs from the F1 hybrids of these two strains are unresponsive to T-mediated 3β-HSD repression. The modifier is not X-linked as there is no difference in T-response in LCs from the reciprocal crosses between C57BL/6 and C3H/HeJ strains, thus ruling out polymorphisms in *Ar* as the modifier. However, the long-term repression of 3β-HSD activity by T appears to be at the transcriptional level *(113)*. After 3 d of T treatment, C57BL/6 LCs show

a severe decrease in *Hsd3b* mRNA, followed by a decrease in protein by day 6 of the treatment. Surprisingly, Hsd3b protein levels are unaffected even on day 4 of T treatment despite dramatic reductions in mRNA on day 3, suggesting a slow turnover rate of this enzyme.

Feedback of T on steroid synthetic gene expression is not limited to *Hsd3b*. After 2 d of culture with T, 8-Br-cAMP-stimulated CYP17A1 expression and activity was decreased in primary mouse LC culture *(114)*. The authors of this study show that the change in expression of CYP17A1 protein is because of a change in the *de novo* protein synthesis rate and not O_2-mediated degradation, as was demonstrated in previous investigations *(115,116)*. Like *Hsd3b*, T is capable of repressing the level of mRNA synthesized under cAMP induction *(111)*. T-mediated repression of *Cyp17a1* expression is most likely mediated by direct interaction of AR with the *Cyp17a1* promoter *(117)*. Using the mouse Leydig tumor line MA-10, Burgos-Trinidad and colleagues demonstrated that a 346 bp fragment from the *Cyp17a1* promoter was sufficient to impart both cAMP and DHT responsiveness to a chloramphenicol acetyltransferase reporter construct. Using DNaseI footprinting, it was shown that baculovirus-produced AR binds specifically to sequences present in the 346 bp fragment.

The *Steroidogenic acute regulatory protein (Star)* has also been shown to be under negative feedback regulation by T *(118)*. Like both *Cyp17a1* and *Hsd3b*, T-mediated repression occurs at the transcriptional level. The levels of *Star* mRNA in MA-10 cells treated with cAMP were reduced in a dose-dependent fashion by increasing concentrations of DHT. Recombinant Müllerian-inhibiting substance (MIS) and cAMP induced higher levels of *Star* mRNA synthesis than cAMP. The MIS-enhanced levels of *Star* transcript were diminished by the addition of 100 n*M* DHT. A 966 bp fragment of the *Star* promoter contained *cis*-acting elements, which mediated all of these transcriptional effects. Within this fragment are three androgen response element consensus half-sites, although, the necessity of these androgen response element half-sites for T-mediated repression has not been demonstrated. The effect of T on *Star* transcript levels was confirmed in vivo with both hypophysectomized mice and mice treated with the GnRH-antagonist acyline. In either model, T was able to repress hCG-stimulated *Star* mRNA levels. Androgenic repression of *Star* and *Cyp17a1* expression was independently confirmed in neonatal mice treated with TP *(13)*. Repression of these transcripts by TP in the neonatal testis suggests that the short feedback regulation mechanism described in earlier studies is functional in murine FLCs.

LC NEOPLASIA IN AR MUTANTS

Most individuals with AIS undergo orchidectomy because of the high incidence of neoplasia in cryptorchid testis. An early estimate of rates of neoplasia in AIS patients was made by Morris *(28)*, based on published case reports from 1817 to 1950, which were likely to be cases of AIS. In this survey, only three cases from individuals under the age of 30 were presented with tumors upon histological evaluation of the testis. However, in 50 cases of AIS patients above the age of 30, the rate of neoplasia was 22% *(119)*. Most of these cases were seminomas (i.e., germ cell tumors), but some were likely Sertoli cell adenomas. Less frequently observed in AIS patients are LC tumors. In more recent review of 43 cases of AIS, only a single Leydig tumor was found among 18 tumors *(39)*. A unilateral LC tumor identified in a 30-yr-old AIS patient was associated with elevated levels of urinary 17β-ketosteroids *(120)*. This is in contrast to another report of a 30-yr-old AIS patient with unilateral LC tumor who demonstrated normal levels of urinary steroids *(121)*. In both of these cases, there is no discussion of tumor size or serum LH levels, so no clear conclusion can be drawn about the steroid output of these tumors. However, in one case of a 73-yr-old patient with AIS who presented with a LC tumor, LH, FSH, and T levels were all within the normal range for males. A LC tumor has also been reported in a case of Reifenstein's syndrome *(122)*. Similar to the previously described patient, high levels of serum LH, FSH, and T were observed. Taken together, these reports suggest that LC tumors in AIS patients can have increased steroidogenic potential, but not in all cases.

The etiology of LC tumors in individuals with AIS is unclear. It has been suggested that high levels of LH are responsible for hyperproliferation and subsequent transformation of LCs in these patients. Some credence to this hypothesis is lent by a recent report of a spontaneous activating mutation in the LH receptor leading to a Leydig tumor in a 3.5-yr-old boy *(123)*. Similar somatic mutations had been previously identified in other boys with virilizing LC tumors *(124)*. Experimental data from the rat show that high levels of LH signaling can induce tumor formation *(125)* and LC tumors can be induced by estrogen in mice *(126)*. The high levels of estrogens produced by the AIS testis make estrogenic induction of LC tumors plausible.

Spontaneous LC tumors also appear in rodents with *Ar* mutations. In the Tfm rat, LC tumors are made up of large polyhedral cells with abundant SER, and small fibroblast-like cells, which are possibly PLCs *(127)*. These LC tumors produce a profile of steroids similar to that in the Tfm testis: high levels of A and relatively small amount of T. The tumor cells responded to LH in culture by increasing T secretion and DNA synthesis. In *Tfm/y* mice, LC tumors are characterized by normal-looking LCs interspersed with groups of large lipid-laden cells *(128)*. Smaller, 3β-HSD-positive cells were observed along the periphery of the tumor *(129)*. Mice with these tumors had serum levels of E_1 and E_2, which were elevated more than their nontumor-bearing *Tfm/y* littermates, but their T levels were still fivefold lower than wild-type mice. When cultured, the Leydig tumor cells from *Tfm/y* mice were stimulated to proliferate by E_2 but not by LH *(129)*. However, the combination of LH and E_2 stimulated significantly higher rates of proliferation than E_2 alone. Although, these results do not reveal the cause of LC neoplasia in the absence of AR, they do show that the endocrine environment of the *Tfm/y* testis supports the growth of LC tumors.

CONCLUSIONS

The AR is required for the normal function and development of LCs. AR is expressed in the interstitial cells of the fetal testis, although, it is unclear if these AR-positive cells are LCs. AR is clearly expressed in the adult generation of LCs, where it is required for their normal development. The developmental requirements for AR are illustrated by LC defects in *Ar* mutants. These mutants fail to acquire normal adult patterns of steroidogenesis, morphological development, and marker gene expression. In the adult, AR participates in the regulation of T-synthesis at the transcriptional level by repressing the expression of key steroidogenic genes. Finally, mutations in *Ar* can, by an unknown mechanism, lead to spontaneous LC neoplasia.

Many outstanding questions remain in the study of AR function in LCs. For instance, what is the mechanism by which AR promotes LC maturation? Is it simply a matter of activating genes, which have already been identified, or is there a more complex transcription network that is set in motion by AR? How does AR mediate negative feedback regulation of steroidogenesis? In at least one case, AR has been shown to bind directly to a steroidogenic gene's promoter. Is this a common mechanism for all steroidogenic genes, or only a subset? What cofactors are involved? Why do AIS patients have elevated levels of aromatase activity? Will androgenic regulation extend to other metabolic pathways in the LC? Answering these important questions will certainly be a part of the advancement of the field of Leydig cell biology.

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V Physiology and Applied Aspects

25 Fluid Exchange and Transport of Hydrophilic Factors in the Testis

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SUMMARY

The fluid environment of the Leydig cells is discussed in relation to the structure of the interstitial tissue of the testis, the composition and volume of interstitial extracellular fluid, the unusual properties of the endothelial cells, and vascular permeability in the testis.

Key Words: Endothelial cells; hormone transport; interstitial extracellular fluid; testis; testicular lymph; vascular permeability.

INTRODUCTION

As the Leydig cells are located in the interstitial tissue between the seminiferous tubules, their fluid environment is interstitial extracellular fluid (IEF), which is quite different in composition from the fluid found inside the tubules (1). It is presumed that IEF is derived by filtration at the arterial ends of the testicular capillaries, which do not penetrate the tubules. The composition of IEF can be influenced by secretions from cells in the interstitial tissue and the tubules, and fluid leaves the interstitial tissue either at the venous ends of the capillaries or via lymphatic vessels present in the interstitial tissue and in the capsule.

FLUID ENVIRONMENT OF THE LEYDIG CELLS

Structure of the Interstitial Tissue of the Testis

The microscopic anatomy of the interstitial tissue varies widely between the different species of mammals *(2–4)*. In most rodents, the Leydig cells lie in clusters surrounded by large fluid-filled lymphatic spaces. In human and domestic ruminants, the space between the tubules is filled with loose connective tissue, in which discrete lymphatic vessels are found. In pigs, horses, and some marsupials as well as in some Australian rodents with comparatively small testes *(5,6)*, the tubules constitute a smaller fraction of the tissue, and the abundant interstitial tissue contains large numbers of closely packed Leydig cells, with very inconspicuous lymphatic vessels *(4)*.

Composition of Interstitial Extracellular Fluid

In pigs, horses, sheep, and cattle, lymph can easily be collected through a catheter in a lymphatic vessel in the spermatic-cord *(7–9)* and composition of this fluid probably reflects that of the interstitial extracellular fluid (IEF) from which it is derived. Lymph from the spermatic-cord of rams and pigs is very similar to blood plasma in its ionic composition and has about two-thirds as much total protein. In lymph from the spermatic-cord in rams, the ratios of the concentration of the various immunoglobulin (Ig)G types to albumin is lower than in plasma, and comparable with lymph from other regions of the body *(10)*. Free steroid levels are comparable with those in testicular venous blood plasma *(7)*, whereas conjugated steroids, such as oestrone sulfate and dehydroepiandrostenedione sulfate, which are much less lipophilic than free steroids, are many times higher than in testicular vein blood *(11,12)*.

In rodents, the lymphatic vessels in the spermatic-cord are too small to cannulate, and lymph from the thoracic duct *(13)* includes fluid derived from many other tissues in the animal as well as the testes. Several techniques have been devised to collect fluid from the lymphatic spaces in the testis itself. The earliest, but least satisfactory, so-called "drip" technique involves removing the testis from the animal, and placing it in a glass vessel in the refrigerator overnight after nicking the capsule at one end *(14,15)*. A modification of this technique, which gives more reasonable values for the testosterone levels, involves suspending the testis by a suture through

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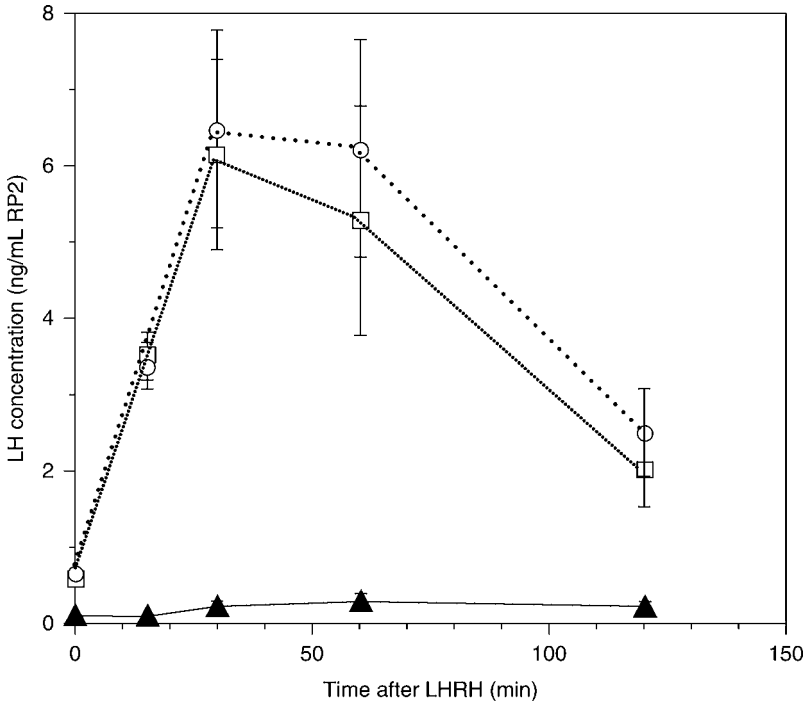


Fig. 1. The concentrations (ng Reference Preparation 2 (RP-2)/mL) of LH in blood plasma from the inferior vena cava (open squares) or testicular veins (open circles), and in IEF, collected from the testes of adult rats by mannitol irrigation, at various times after intravenous injection of GnRH (1.5 or 0.5 μg). Values shown are means ± SEM for eight animals at times 0 and 15 min, and four animals at the other times. Data from ref. *20*. Note that there was no significant differences between the two sources of blood plasma, indicating that the testis did not appear to take up any LH, and the concentration in IEF was lower than in blood before injection of GnRH, and showed a much smaller absolute and proportional increase after the injection.

the capsule at the other end to the nick and centrifuging the suspended testis very gently for a short time *(16–18)*. Techniques for collecting the least contaminated IEF for analysis involves the use of a push-pull cannula *(19)* or opening the capsule of the testis and irrigating the interstitial tissue with ice-cold isotonic mannitol, which washes the IEF out of the testes. Assuming that the sodium concentration of the IEF is the same as blood plasma, as per good evidence, and by knowing the volume of mannitol used, it is possible to calculate the extent of dilution of the IEF, and therefore, the concentrations in the original fluid and its volume *(20)*. IEF in very small amount can also be collected by micropuncture after opening the capsule in anesthetized rats *(21)*, although, the small volumes collected in this way limit the possible analyses.

Fluid collected by the "drip" technique from rats contains appreciably more potassium and very much more testosterone than testicular venous blood plasma, indicating that the fluid collected in this way is contaminated with cell contents, or that the Leydig cells continue to leak or secrete testosterone into the fluid during the collection *(22)*. The total protein concentration is only slightly less than blood plasma *(23)*, a finding, which is supported by the appearance of the contents of the lymphatic sinusoids in the rat testis in frozen section examined by electron microscopy and from the distribution of fluorescent-labeled antibody to albumin *(2,24)*. IgG levels in rat IEF are about two-thirds of those in blood plasma, higher than elsewhere in the body *(23)*, in contrast to the situation in sheep *(10)*. Surprisingly, luteinizing hormone (LH), which is a smaller molecule than albumin, is present in IEF at concentrations about one-fifth of those in blood plasma, and furthermore, the concentration of LH in IEF does not rise significantly in the 15 min following an injection of gonadotropin-releasing hormone (GnRH), in a dose sufficient to raise blood levels of LH by about 10 times by that time (Fig. 1), when the Leydig cells had already increased there content of testosterone. These findings support the earlier observations that when iodinated LH was infused into the testicular artery of rats, the concentration in IEF at the end of a 15 min infusion was only 4.2% of that in venous blood plasma *(21)*. Either the Leydig cells respond to changes in LH which were too small to detect or another cell type was sensing the rise in LH and then influencing the Leydig

cells *(20,25)*. The obvious candidates are the endothelial cells, which have been shown to have receptors for LH *(26)* and it is interesting that cocultures of isolated endothelial and Leydig cells from rat testes produced more testosterone than either cell type alone *(27)*. IEF also contains an GnRH-like substance *(28–30)*, which might influence steroid secretion by the Leydig cells, and IEF contains more androgen-binding protein *(17)*, arginine vasopressin *(31)*, β-endorphin, and adrenocorticotropic hormone (ACTH) *(32)* than testicular venous blood. The concentration of β-endorphin in IEF is increased after intraperitoneal injections of ethanol, which also produce falls in testosterone concentrations *(33)*. An interleukin-like substance can also be detected in IEF *(34,35)*. Some amino acids are present in IEF in concentrations, which differ slightly, but significantly from venous blood plasma *(36)*.

Volume of IEF

An estimate can be made of the volume of the IEF collected by either the drip or centrifuge techniques, and values obtained correspond reasonably with the volume of distribution of suitable marker, such as iodinated albumin or Cr-labelled ethylene diaminetetraaceticacid *(37–39)*, which do not enter cells or the fluid in the lumina of the seminiferous tubules. The volume of IEF is determined by permeability of the microvasculature of the testis, the difference in protein concentration between blood plasma and IEF, but also by capillary and tissue pressures, so IEF volume should not be taken as an index just of permeability. IEF volume in rats is decreased after hypophysectomy or treatment with estradiol *(40)* or ethanol *(33)* or for up to 10 d after removal of the Leydig cells with ethane dimethane sulfonate (EDS) *(41–44)*, although, at longer times after EDS, the volume of IEF appears to increase *(43)*. The changes after EDS can be eliminated by treating the animals with testosterone, although testosterone implants in control rats caused a fall in IEF volume *(45)*. An intratesticular injection of a GnRH agonist (GnRH-A) caused 50% reduction in IEF volume within 2 h *(46)*, whereas treatment of rats with a GnRH-A implant and an antiandrogen, which caused testis weight to fall, led to a reduction in IEF volume expressed per testis, but not per unit weight of testis *(47,48)*. On the other hand, other studies suggest that a subcutaneous injection of GnRH-A, which caused a rise in plasma LH levels also caused a rise in volume of IEF *(32,49)*. Injection of LH itself *(50)* or human chorionic gonadotrophin (hCG) in rats leads to a large rise in IEF volume, beginning about 8 h after injection and peaking at about 24 h *(15,37)*. This rise depends on the presence of Leydig cells, but is not mediated by androgens, prostaglandins, histamine, or bradykinin *(51,52)*, but might involve 5-hydroxytryptamine. The injection of lipopolysaccharide to produce a systemic inflammation caused a large fall in IEF volume which was accompanied by a fall in testosterone in IEF *(44,53)* and blood *(54)*.

Any treatment, which causes regression of spermatogenesis and shrinkage of the seminiferous tubules seems to lead to an increase in IEF volume. This has been seen after treatment of rats with methoxyacetic acid *(55)*, busulfan *(56)*, procarbazine *(57)*, or 1,2-dibromo-3-chloropropane *(58)*, local heating of the testes *(39,47,55)*, cryptorchidism *(59–61)*, X-ray irradiation *(38,62)*, or efferent duct ligation *(60,63)*. However, this is probably because of a decrease in tissue pressure resulting from shrinkage of the tubules within the relatively inelastic capsule. The testicular edema, which follows irradiation can be reduced by treatment of the rats with an antagonist to GnRH. This effect is partially blocked by implants of testosterone, but increased by estradiol *(62)*.

The IEF/plasma ratio of IgG has been used as a measure of permeability of the microvasculature of the testis. Following cryptorchidism, there is an increased IEF volume, with no change in the IEF/plasma ratio for IgG *(23)*. However, in this same study, it was found that the vehicle dimethylsulphoxide eliminated the IgG concentration gradient between IEF and plasma, indicating an increase in microvascular permeability. In a later study, it was found that GnRH-A injection into the testis, although reduced IEF volume, had no effect on the IgG IEF/plasma ratio, whereas this was increased by 2 h after a subcutaneous injection of hCG, but had returned to control levels by 24 h, when fluid accumulation was maximal *(46)*.

ENDOTHELIAL CELLS IN THE TESTIS

It is assumed, without any direct evidence, that IEF in the testis is formed as in other tissues, by filtration of a low-protein fluid at the arterial ends of the capillaries where pressure inside the vessel exceeds that in the tissue. Resorption of the majority of the filtered fluid, but none of the protein, occurs at the venous end of the capillaries, where the pressure inside the vessel is less than in the tissue. The small amount of protein, which enters the interstitial space is removed, with some of the fluid in the lymph *(64)*. Pressure inside capillaries in the capsule of the hamster testis is lower (10 mmHg) than arterial pressure (89 mmHg), but it rose appreciably when venous pressure was elevated.

There was a very small pressure gradient between arterioles and venules, the pressures in which were very similar to that in the capillaries *(65,66)*. However, the situation might be quite different in capillaries in the parenchyma, which appear to be much less permeable *(67)*. Little information is available about pressure in the interstitial tissue, except that contractions of the capsule appear to be important in some species *(68,69)* and the pressure is higher in cryptorchid testes *(70)*.

The endothelial cells lining the capillaries are obviously of prime importance in regulating the filtration and reabsorption of fluid and proteins, and it was shown many years ago that certain dyes did not readily pass from the blood stream into the interstitial tissue of the rat testis *(71)*. The endothelial cells of the testis, in contrast to those from other endocrine tissues, are unfenestrated *(2,72–74)*, although in human testes, some of the capillaries penetrating the lamina propria of the seminiferous tubule do appear to have fenestrations *(75)*. The endothelial cells of the rat testis show a high rate of proliferation *(76)*, which is reduced if Leydig cells are eliminated with EDS, but is not restored by testosterone treatment *(77,78)*. An angiogenic mitogen (endocrine gland vascular endothelial growth factor, selective for endothelial cells from a number of endocrine tissues has recently been identified *(79)*, and receptors for its homolog Bv8 are present in testicular endothelial cells *(80)*. Ordinary Vascular Endothelial Growth Factor (VEGF) is secreted by Leydig cells and its receptors are present on testicular endothelial cells *(81–83)*. These cells also have rece tors for endothelin *(84,85)* and glial cell-line-derived neurotrophic factor *(86)* and have lower densities of endothelial vesicles than those from other tissues, except brain *(87)*, suggesting that vesicular protein transport is low in both testis and brain. Endothelial cells are the only cells in the testis to express claudin-5, a protein constituent of tight junction strands, whereas occludin, another protein associated with tight junctions is found in endothelial cells as well as the Sertoli cells *(86)*. The endothelial cells of the rat testis have a number of other unusual characteristics, many of which they share with endothelial cells in the brain, which constitute the blood–brain barrier. Both cell types have a high content of γ-glutamyl transpeptidase *(88,89)*, a protein, which is usually associated with amino acid transport. A transport system for amino acids, in particular leucine has been demonstrated in the endothelial cells of the larger vessels of the testicular microvasculature *(90)*. This carrier has K_M and V_{max} values similar to those for brain, and lower than the values for other tissues. The large amino acid transporter 1, is also present in rat testis as well as brain and heart, but not other tissues *(91)*. Endothelial cell in the testis, also, contain an endothelial barrier antigen, which had previously been thought to be confined to vessels in the central nervous system *(92)*. Testicular endothelial cells also contain a glucose transporter isoform GLUT-1, usually, associated with brain and retina, and also the mutltidrug resistance protein, P-glycoprotein *(89)*. This transporter is located only on the luminal surface of endothelial cells in the brain, but on both luminal and abluminal surfaces in the testis. This localization suggests that the protein in the testis is involved in excluding P-glycoprotein substrates from the endothelial cells in the testis, rather than protecting the tissue from circulating lipophilic molecules as in the brain *(93)*. However, pharmacological inhibition of P-glycoprotein enhances the penetration of HIV-protease inhibitors into the testis as well as the brain *(94)*, and in mice, in which the *mdr1a* gene for P-glycoprotein has been knocked out, the testis and brain concentrations of ivermectin, digoxin, cyclosporin A, ondasetron, and loperamide were higher than in wild-type mice *(95–97)*. Furthermore, in mice in which both *mdr-1a* and *mdr-1b* P-glycoprotein genes were knocked out, the penetration of the steroids corticosterone, cortisol, aldosterone, and progesterone into the testis was enhanced *(98)*, suggesting that these proteins might also be involved in steroid movements in the testis.

VASCULAR PERMEABILITY

The entry of hydrophilic molecules into the testis is determined by two factors, permeability of the endothelial cells and net fluid flux. Permeability is bidirectional and is a property of the endothelial cells, but net fluid flux involves unidirectional transport of substances from the vessel lumen into the tissue along with the fluid filtered from the capillaries, and involves features of the interstitial tissue as well as the walls of the blood vessels, and probably also changes in capillary and/or tissue pressures, and should probably be referred to as apparent permeability. It is recognized that in other tissues, the nature of the extravascular tissue is the rate-limiting step in plasma to lymph transport of albumin *(99)*.

Measurement of permeability of just the endothelial cells or true permeability involves the injection of a suitable marker into the arterial inflow to the testis, with an estimate of the proportion of the dose, which lodges in the tissue during a single pass of the marker. This is best done using an isolated perfused testis, but it can be also done *in vivo* if the marker is injected into the arterial

supply to the testis and the tissue is removed before appreciable recirculation occurs. Using isolated perfused rat testes, it has been found that 1.27% of a dose of iodinated albumin is taken up in one passage *(100)*, and with ram testes *in vivo*, about 3.1% of an injection into the testicular artery on the surface of the testis is retained in the tissue *(4)*. Permeability calculated from these figures and flow rates give a value for permeability in both species of about 3μL/g/min *(4)*. The permeability of isolated perfused rat testes to smaller hydrophilic markers is higher (850, 1480, and 2230 μL/g/min for vitamin B_{12}, Cr-ethylene diaminetetraaceticacid, and sodium, respectively *(100)*. If allowance is made for the smaller surface area of the microvasculature in the testis, compared with other tissues for which similar measurements have been made, it appears that the vessels in the rat testis are very much permeable to these markers than other tissues with unfenestrated capillaries.

Apparent permeability can be estimated from the rate at which the volume of distribution of a suitable marker such as iodinated albumin or IgG approaches its equilibrium value. In rats, values of about 0.3 μL/g/min for albumin can be obtained *(101,102)*. Similar values have been obtained in rats for iodinated sheep IgG *(45,67)* with slightly higher values for the F_{ab} fragment of IgG *(103)*. As these values are clearly less than that obtained for true permeability of the endothelial cells, it is clear that other components of the interstitial tissue are influencing the rate at which the marker enters the tissue. Apparent permeability of rat testes, measured by the rate of accumulation of albumin or IgG in the tissue, increases at about the age of 30 d *(45,67,102)* and there is a massive increase after the injection of hCG *(37)*, which leads to an accumulation of IEF (*see* p. 367). In cryptorchid testes, apparent permeability is lower than normal, but there is a larger increase in the volume of IEF after hCG *(59,61)*. Injection of cadmium salts, in doses, which are not toxic to other tissues, leads to a massive increase in apparent permeability, so large that it leads to a catastrophic fall in blood flow and necrosis of the tissue *(104)*. Cadmium salts also cause an increase in true permeability of the endothelial cells of the rat testis *(105)* and a rise in pressure inside the testis *(69)*, but there is no change in true permeability corresponding to the increases in apparent permeability seen after hCG or during puberty *(105)*. In contrast to the effects seen in other tissues, histamine or serotonin (5-hydroxytryptamine) have no effect of apparent permeability of rat testes *(106)*.

Increases in apparent permeability would also lead to increased lymph flow, which might be important in regulating the secretion of hydrophilic hormones, peptides, and conjugated steroids, from the testis, as it has been shown that about 70% of a bolus of iodinated albumin injected into the interstitial tissue of a rat, ram, or boar testis can be recovered in lymph from the thoracic duct or spermatic-cord *(107,108)*. Increased fluid turnover in the interstitial tissue might also lead to wash-out of important paracrine messengers carrying information from cell type to another in side the testis. Increases in venous and hence capillary pressure, as seen in varicocele *(109–111)*, space flight *(112)*, or caudal elevation *(113)*, would also lead to increased fluid turnover, which could also have similar effects.

CONCLUSIONS

The fluid environment of the Leydig cells is obviously important in regulating their activity, and the nature and amount of the fluid in the interstitial tissue (IEF) is affected by a number of factors, some within the testis and some in the circulating blood. The recently raised possibility *(62)* that edema or increased amount of IEF might affect differentiation of spermatogonia following radiation emphasizes the potential importance of this fluid. It will be important to determine if this is a direct effect of the increased amount of fluid, or whether this and the effect on the spermatogonia both result from something else. Techniques are now available for measuring many features of the endothelial cells and other components of the interstitial tissue, and some interesting results can be anticipated in the near future.

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26 Immortalized Leydig Cell Lines as Models for Studying Leydig Cell Physiology

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SUMMARY

This chapter provides a brief overview of the properties of various immortalized Leydig cell lines and their usefulness in the study of Leydig cell physiology.

Key Words: Cell culture; Leydig cell; Leydig cell tumors; lutropin/choriogoandotropin receptors; steroidogenesis; transfection.

INTRODUCTION

The use of primary cultures for the study of Leydig cell physiology is particularly difficult because of the low abundancy of Leydig cells in the testes. Although highly enriched (~90%) populations of viable Leydig cells can be obtained from rat or mice testes, but the methods needed to isolate them are rather laborious (*see* e.g., in refs. *1–7*). Moreover, primary cultures of rodent Leydig cells become unresponsive to gonadotropins, and loose their ability to synthesize steroids after a few days in culture *(1,4–12)*.

The availability of transplantable mouse and rat Leydig cell tumors that retain gonadotropin-responsiveness made it easy and possible to isolate large numbers of relatively homogenous populations of Leydig tumor cells, but their hormonal responsiveness was influenced by the host and by the age of the tumor *(13–18)*. Immortalized Leydig cell lines obtained from these transplantable tumors, or from a Leydig cell tumor that arose in transgenic mice *(19)* obviates many of these problems, and provide valuable models for studying Leydig cell function, and regulation in cell culture. This chapter provides an overview of the various immortalized Leydig cell lines available and their properties. Another review on this topic has been published recently *(20)*.

STRATEGIES USED TO IMMORTALIZE LEYDIG CELLS

Leydig Tumor Cells

The majority of immortalized Leydig cell lines available (Tables 1 and 2) have been obtained by adapting rat or mouse Leydig cell tumors to grow in culture *(19,21–26)*. The first two clonal lines of cultured Leydig tumor cells were established by Shin and colleagues *(23,24)*. The I-10 cells are a clonal cell-line derived from a mouse Leydig cell tumor (designated H10119). These cells were adapted to grow in culture using the alternate culture animal passage technique initially described by Buonassisi et al. *(27)*. This technique, was shown to improve the growth and retention of differentiated function of mass cultures of malignant endocrine cells *(27)*. Shin et al. *(23)* also established an additional clonal strain of rat Leydig tumor cells (designated R2C), which did not require the use of the culture animal passage technique. The I-10 and R2C cell lines are available from the American Type Culture Collection (ATCC) (CCL-83 and CCL-97, respectively). LC-540 cells are an additional clonal line of rat Leydig tumor cells *(26)* that are also available from the ATCC (CCL-43). This cell line was established from a transplantable rat Leydig cell tumor known as H-540 *(18)*.

MA-10 *(21)* and the closely related MLTC-1 cells *(22)* are by far the best characterized and more widely used lines of cultured Leydig tumor cells (Tables 1 and 2). They are clonal cell lines that were independently derived from the M5480 tumor, a hormonally responsive mouse Leydig tumor, which originated spontaneously in a C57Bl/6J mouse *(13–17,28,29)*. Two variants of the M5480 tumor were later identified: M5480A, which synthesizes progesterone and testosterone, and M5480P, which synthesizes mainly progesterone *(17,30)*.

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Table 1
Cell Biology of Commonly Used Immortalized Leydig Cell Lines

Cell line	*Species, strain*	*Name of tumor*	*Recommended culture medium*	*Doubling time (h)*	*Modal chromosome number*	*References*
I-10	Mouse, BALB/cJ	H10119	Ham's F10 (82.5%) Horse serum (15%) Fetal calf serum (2.5%)	84	41	*24*
R2C	Rat, Wistar-Furth	NR[a]	Ham's F10 (82.5%) Horse serum (15%) Fetal calf serum (2.5%)	NR[a]	43	*23*
LC540	Rat, Fisher	F540/H540	Eagle's MEM (90%) Fetal calf serum (10%)	NR[a]	42	*18,26*
MA-10	Mouse, C57Bl/6	M5480P	RPMI-1640 with 20 m*M* Hepes[b] (85%) Horse serum (15%)	18–25	102	*21*
MLTC-1	Mouse, C57Bl/6	M5480P	RPMI-1640 (90%) Fetal calf serum (10%)	35–40	95	*22*
BLT-1	Mouse, C57Bl/6	NR[a]	DMEM/F12 with 20 m*M* Hepes (90%) Heat inactivated fetal calf serum (10%)	36–48	NR	*19*

[a]NR, not reported.
[b]Originally this cell line was maintained in Waymouth's MB752/1 medium but they can be maintained equally well in RPMI-1640. The switch to RPM1-1640 was necessitated by the fact that Waymouth's MB752/1 medium is no longer available from commonly used suppliers.

Table 2
Functional Properties of Commonly Used Immortalized Leydig Cell Lines

					Steroidogenic response to			
Cell line	*hCG receptors*	*hCG-stimulated cAMP synthesis*	*hCG-stimulated IP accumulation*	*Major steroids*[a]	*hCG*	*Cholera toxin*	*cAMP*	*References*
MA-10	Yes	Yes	No	P, 20-OHP	Yes	Yes	Yes	*21*
MLTC-1	Yes	Yes	No	P, 20-OHP	Yes	Yes	Yes	*22*
BLT-1	Yes	Yes	NT[b]	P	Yes	NT	NT	*19*
I-10	No	NT[b]	NT[b]	P, 20-OHP	No	Yes	Yes	*24*
R2C	?[c]	No	NT[b]	P, 20-OHP	No	No	No	*23,57,68,69*
LC-540	NT[b]	NT[b]	NT[b]	T, E2	NT[b]	NT[b]	NT[b]	*26*

[a]P, progesterone; 20-OHP, 20α-hydroxyprogesterone; T, testosterone; E2 17β-estradiol.
[b]N.T., not tested.
[c]?Conflincting data have been reported.
See text and references for details.

MA-10 cells, which are gonadotropin responsive, as well as several unresponsive clonal cell lines (notably MA-14) were established in this laboratory *(21)* by using the alternate culture animal passage technique *(27)* and the M5480P tumor. MLTC-1 cells were also derived from the M5480P tumor by direct culturing methods *(22)*. MA-10 cells are not available from the ATCC but I have distributed them to over 500 investigators worldwide*. However, MLTC-1 cells are distributed by the ATCC (CRL-2065).

*After the publication of the initial paper describing MA-10 cells I tried to deposit this cell line with the ATCC. They rejected that submission, however, because they thought there would not be enough interest in these cells. I never tried to submit them again.

More recently a new Leydig tumor cell line, designated BLT-1, was established from a Leydig cell tumor present in a founder C57Bl/6 transgenic mouse expressing the SV40 T-antigen driven by the inhibin-α promoter *(19)*. Like MA-10 and MLTC-1 cells, BLT-1 cells express the endogenous luteinizing hormone receptor (LHR) and respond to human chorionic gonadotropin (hCG) with increases in cAMP and progesterone accumulation *(19)*.

A few other mouse Leydig tumor cell lines have been adapted to multiply in culture, but their functional properties are poorly characterized. These include B-1-A-2 cells, which were derived from an estrogen-responsive mouse Leydig cell tumor *(25)*, TTE1 cells which were established from transgenic mice harboring a temperature sensitive SV40 T antigen *(31,32)*, and D-4 cells, which were established from transgenic mice harboring the large T-antigen of the polyoma virus driven by the metallothionein-1 promoter *(33)*.

Somatic Cell Hybridization

In an attempt to establish a clonal Leydig cell line capable of synthesizing testosterone, Finaz and colleagues *(34)* hybridized MA-10 cells with freshly isolated mouse Leydig cells. Subcloning of the resulting hybrids led to isolation of the K9 mouse Leydig cell line, a cell line, which is capable of synthesizing testosterone. One disadvantage of this cell line is that after 2–3 mo in culture they appear to revert to the phenotype of the MA-10 cells, thus, necessitating routine subcloning *(34)*.

Direct Culturing of Testicular Cells

The TM3 cells were cloned from a culture of mixed mouse testicular cells *(35)*. TM3 cells are listed as a Leydig cell line by the ATCC (CRL-1714) but their functional properties and hormonal responsiveness are not well characterized. Other than the fact that this cell line was derived from a mixed culture of testicular cells, there are few (if any) indications that TM3 cells are indeed Leydig cells.

Transformation of Primary Cells

Two immortalized rat Leydig cell lines (NWL2 and NWL15, *see* ref. *36*) were also obtained by transfection of primary cultures of rat Leydig cells with the transforming region of simian virus DNA. These cell lines, also, have not been fully characterized with respect to gonadotropin responsiveness and steroidogenesis.

PROPERTIES OF IMMORTALIZED LEYDIG CELL LINES

Lutropin/Choriogonadotropin Receptors

Binding of radiolabeled hCG or hLH to MA-10, MLTC-1, and BLT-1 cells can be readily demonstrated. These cell lines bind LH/CG with the expected high affinity (100–500 pM), and express 1000–40,000 receptors/cell *(19,21,22,37–47)*. The presence of endogenous LHR in one or more of these three cell lines has also been demonstrated by crosslinking of radiolabeled hCG *(22,48)*, by immunoprecipitation of the hormone–receptor complex using an antibody to hCG *(49)*, by detection of LHR transcripts using Northern blots or solution hybridization assays *(19,50–52)*, and by measuring the transcription of the endogenous LHR gene *(52)*.

A few years ago three important changes were noticed in the behavior of MA-10 cells. First, instead of being firmly attached and having an epithelial-like morphology they became more round, displayed loose attachment to culture vessels and appeared to form clusters. Second, their ability to proliferate in culture diminished and third, the density of cell surface LHR declined dramatically from the initial estimates of 5000–20,000 receptors/cell (reviewed in ref. *53*) to 600–1000 receptors/cell *(47)*. Although the reasons behind these phenotypic alterations are not known with certainty it is suspected that they are related to recent changes in the formulations used to treat the surface of the plasticware used for cell culture. This conclusion was made because the changes described earlier can be readily detected upon thawing MA-10 cells, which were frozen in 1981 and maintained in liquid nitrogen since then; and because two of these phenotypic alterations (i.e., the change in cell morphology and proliferation capacity) can be readily reversed by coating the plasticware used to culture the cells with gelatin (described in ref. *47*).

However, the decrease in LHR density cannot be reversed by this manipulation, and the reason for this decline remains more of a mystery. It's been known for years that the batch of serum used to culture MA-10 cells can have dramatic effects on LHR density and care has been taken in pretesting several batches of serum before using them. Recently, however, not a single batch of serum (regardless of vendor) that supports the density of LHR previously reported in MA-10 cells was found. Because MA-10 cells cultured in gelatinized plasticware can express the LHR when transfected with an expression vector driven by a strong heterologous promoter *(47)* it is believed that the

decrease in the expression of the endogenous LHR in MA-10 cells was caused by contaminants present in the cell culture plasticware or in the preparations of gelatin used to coat the plasticware. It was already known that some hormones such as epidermal growth factor as well as second messenger analogs such as 8Br-cAMP and phorbol esters can decrease the density of endogenous LHR in MA-10 and MLTC-1 cells by decreasing the transcription of the endogenous gene *(50,52,54)* and unknown contaminants in the preparations of gelatin could have a similar effect. In addition cell culture plasticware is known to contain steroid-like compounds *(55,56)*, which could have a similar effect on the expression of the endogenous LHR. Lastly, it is worth noting that in spite of the low density of endogenous LHR currently being expressed by MA-10 cells, these receptors remain functional as judged by the ability of these cells to respond to hCG with increased cAMP and progesterone accumulation *(47)*.

I-10 and R2C cells were initially described as being unable to bind hCG. In more recent years; however, there has been at least one published report indicating that radiolabeled hCG binding can be detected in R2C cells *(57)*. It was reported that R2C cells could bind hCG *(58)*, but later it was found that this was a result of contamination of R2C cultures with MA-10 cells (unpublished observations).

Second Messenger Cascades

The activation of second messenger cascades is one the most proximal functional events of LH/CG binding that have been measured in immortalized Leydig cells. There are many studies documenting an LH/hCG-induced increase in adenylyl cyclase activity or cAMP accumulation in MA-10 and MLTC-1 cells *(21,22,37, 41–44,47,59–67)*. An increase in cAMP accumulation in response to LH/CG in BLT-1 cells has been recently reported *(19)*.

There are no reports available on the effects of LH/CG on cAMP accumulation in I-10 or LC540 cells. Two different groups of investigators have reported that hCG is unable to increase cAMP levels in R2C cells *(68,69)*. As R2C cells respond to cholera toxin or forskolin with increased cAMP levels *(68,69)*, the lack of response to hCG must be because of the absence of LH/CG receptors, or to a failure of these receptors to activate the Gs/adenylyl cyclase system.

Several studies that have explored the effects of hCG on inositol phosphate accumulation in Leydig tumor cells produced negative results in spite of the fact that the LHR can activate this pathway when expressed in a number of heterologous cell lines *(70–79)*. Inoue and Rebois *(67)* showed that MLTC-1 cells do not respond to hCG with an increase in inositol phosphate accumulation but they responded well to stimulation with AlF^{+4}, a universal activator of G proteins. Likewise, MA-10 cells fail to respond to hCG with increases in inositol phosphates or diacylglycerol accumulation, but they show a robust responses when stimulated with arginine vasopressin *(80–82)*. In retrospect the lack of effect of LH/CG on the inositol phosphate/diacylglycerol pathway in MA-10 and MLTC-1 cells is not surprising, as it is now known that this response is detectable only in cells expressing a high density of LHR and it requires high concentrations of hCG *(70–79)*. As described earlier the density of endogenous LHR in Leydig tumor cells is low and the initial experiments exploring an effect of LH/CG on inositol phosphate accumulation in Leydig tumor cells were done with low concentrations of LH/CG, which are now known to be ineffective in stimulating inositol phosphate accumulation in other cells *(67,80)*. However, the main reason why the endogenous LHR does not support an inositol phosphate response in MA-10 cells is because of the low density of LHR. As shown in a recent report from this laboratory hCG can stimulate the inositol phosphate cascade in transfected MA-10 cells expressing higher densities of the recombinant hLHR *(47)*. To the best of my knowledge the effects of LH/CG on inositol phosphate accumulation in I-10, R2C, LC540, or BLT-1 cells have not been tested.

Steroidogenic Pathway

The hallmark of normal Leydig cell function, the ability to respond to LH/CG stimulation with increased testosterone synthesis, is not retained by any of the immortalized Leydig cell lines described earlier. All of these cell lines can synthesize steroids, but their steroidogenic pathways are truncated in such a way that the major steroids produced (from endogenous precursors) are progesterone and/or 20α-hydroxyprogesterone rather than androgens (Table 2).

The loss of testosterone synthesis in MA-10 cells is owing to a loss of CYP17 (P450c17) expression. MA-10 cells have very low or undetectable levels of P450c17 mRNA *(83,84)*, and they synthesize large amount of progesterone and 20α-hydroxyprogesterone, and minimal or undetectable amount of 17α-hydroxyprogesterone, androstenedione, and testosterone *(21,84)*. This loss of CYP17 expression likely occurred during transformation rather than as a result of adapting the cells to culture, because Leydig tumor cells freshly isolated from the parent (M5480P) tumor also express a steroidogenic

pathway that reflects reduced levels of 17α-hydroxylase activity *(30)*. Because the acute activation of steroidogenesis occurs at the conversion of cholesterol to pregnenolone (review in refs. *85,86*), the lack of CYP17 *per se* should not prevent the activation of steroidogenesis, provided that the other steps of this pathway are functional. This is in fact the case in MA-10, MLTC-1, and BLT-1 cells, which respond to LH/CG and/or cAMP stimulation with a robust increase in progesterone synthesis *(19–22)*.

The major steroids synthesized by R2C and I-10 cells are also progesterone and 20α-hydroxyprogesterone (Table 2), but it is not known if these cells do not express CYP17, or if they express an inactive form of the enzyme. LC-540 cells are reported to synthesize testosterone and estradiol but it is not known whether steroid production is stimulated by gonadotropins or by cAMP *(26)*.

The effects of LH/CG, cAMP analogs and/or cholera toxin on steroid synthesis in MA-10 and MLTC-1 have been documented by many investigators (*see* the following references for a few examples *20,21,37, 61,84,87–97*). An increase in progesterone accumulation in response to LH/CG stimulation in BLT-1 cells has been recently reported *(19)*.

The responsiveness of LC-540 cells has not been tested. I-10 cells do not respond to LH/CG with increased steroid synthesis, but do respond to cAMP analogs and cholera toxin *(24,98)*. R2C cells synthesize large amounts of progesterone under basal conditions, and do not respond with increased steroidogenesis to LH/CG, cAMP analogs, or cholera toxin *(23,68,69,99,100)*. This is most likely due to the constitutive expression of high levels of several proteins used in cholesterol trafficking and utilization *(101,102)*.

Transfectability

MA-10 and/or MLTC-1 cells have been transfected using calcium phosphate *(52,64,66,103)*, by electroporation *(104)* or with cationic lipids such as Lipofectamine® *(47,81,82,105,106)* or Fugene® *(107)*. The efficiency of transfection with Lipofectamine is about 25% *(47)* and this is the method of choice for the expression of recombinant proteins *(47,81,82,108)*. However a systematic comparison of the efficiency of transfection attained with these two cationic lipids has not been performed. It has been found that the expression of recombinant proteins in MA-10 cells is better with an expression vector driven by the elongation factor 1α-subunit promoter rather than the more commonly used cytomegalovirus promoter *(47)*. Maintaining the transfected cells at 30°C (instead of 37°C) after the transfection also enhances the expression of some (but not all) transfected constructs *(47)*.

Although, transfections with cationic lipids have been also used for reporter gene studies *(86,104,109)* the levels of expression attained with a reporter such as luciferase are high enough with the more economical calcium phosphate method *(52,103)*.

Transiently transfected MA-10 or MLTC-1 cells have been widely used in reporter gene studies characterizing the promoters of genes such as the LHR *(52,103,104,110–113)*, steroidogenic factor-1 *(106,114)*, steroidogenic acute regulatory protein *(86,109)* and several steroidogenic enzymes *(85)*.

Transiently transfected MA-10 cells have also been recently used to study the functional properties of the hLHR-wt and some of its naturally occurring mutants *(47,81,82)*. Because MA-10 cells currently have a very low number of endogenous LHR, their use was proposed as a suitable host to examine the functional properties of the hLHR. MA-10 cells provide a more relevant cellular context for the study of hLHR expression than is provided for in heterologous systems such as human kidney 293 cells or monkey kidney COS-7 cells *(47)*. Under optimal conditions the expression of transfected hLHR constructs can be 100–200 times higher than the level of endogenous mLHR currently expressed in MA-10 cells *(47)*.

In fact, MA-10 cells transiently transfected with the hLHR-wt or three of its constitutively active mutants have been successfully used to identify and characterize the G proteins and signaling pathways activated by the LHR *(47,81)*. The data show that the agonist-engaged hLHR-wt, two naturally occurring constitutively active mutants associated with Leydig cell hyperplasia and one constitutively active mutant associated with Leydig cell adenomas activate three (G_s, $G_{i/o}$, and $G_{q/11}$) of the four families of G proteins *(81)*. The data also show that these three mutants of the hLHR can readily activate the cAMP and inositol phosphate signaling cascades as well as steroid synthesis and the phosphorylation of ERK1/2 *(47)*. These results show that the G proteins and signaling cascades activated by constitutively active mutants of the hLHR associated with Leydig cell hyperplasia or Leydig cell adenomas are identical. Moreover they are the same as those activated by the agonist-engaged hLHR-wt *(47,81)*. Gs, of course, mediates the ability of the transfected hLHR to stimulate cAMP synthesis whereas, $G_{q/11}$ mediate the effects on inositol phosphates *(47,81)*. However, the functional consequences of the LHR-mediated activation of $G_{i/o}$ are not known.

MA-10 cells transiently transfected with the hLHR-wt and a variety of other expression vectors, such as those encoding cAMP phosphodiesterase, a protein kinase A inhibitor or dominant negative mutants of Ras, were recently used to define the pathways by which the LHR activates the ERK1/2 cascade *(82)*.

There are a few stably transfected subclones of MA-10 cells, which have been isolated and characterized *(64,66,105,115,116)*. MA10(K3), stably express a mutant form of the regulatory subunit type-I of protein kinase A *(64)*, whereas MA-10(P+29), stably overexpress the wild-type cAMP phosphodiesterase 3 *(66)*. These two cell lines display a partial cAMP-resistant phenotype and have proven useful in the study of the role of cAMP in steroid biosynthesis *(64,66,115)*. Two other subclones of MA-10 cells stably transfected with an antisense vector targeting stathmin, a prominent protein phosphorylated in response to hCG and EGF stimulation *(117,118)*, have been produced in this laboratory *(119)*. These cell lines have reduced levels of stathmin and are useful in the study of the functions of this protein *(119)*. Others also have stably transfected MA-10 cells with temperature sensitive mutants of Src and constitutively active mutants of Ras *(105,116)*. These have been used to study the effects of Src and Ras on LHR-mediated responses *(105,116)*.

CONCLUSIONS

The popularity of clonal strains of Leydig tumor cells to study Leydig cell physiology has continued to increase over the last decade *(53)*. This statement is perhaps the best documented by the fact that 24 yr after its publication, the initial paper describing MA-10 cells *(21)* is among the 50 most cited articles published in *Endocrinology (http://endo.endojournals.org/reports.mfc1.dtl)*. As it is true for all differentiated cells in culture, Leydig cell lines do not retain all the differentiated functions of normal Leydig cells. They do resemble their normal counterparts in many important aspects; however, and offer all the advantages associated with the use of cultured cells. They are a relatively homogeneous population of cells that can be readily propagated in cell culture and are amenable to modern experimental approaches such as transfections. It should be clear from the preceding discussion that when used judiciously, immortalized Leydig cell lines can be successfully exploited to study many aspects of Leydig cell physiology.

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27 Induction of Leydig Cell Tumors by Xenobiotics

Paul M.D. Foster, PhD

SUMMARY

This chapter provides a brief review of the basic biology, mechanisms, and chemical examples of agents known to produce an increased incidence of Leydig cell tumors (LCT) after long-term exposure in experimental animals. The diagnosis of an LCT in such studies is one that relies primarily on the size of the area of LC proliferation and the overwhelming majority of such tumors are benign. LCTs have always been regarded as rare tumors in human although, it might be possible that different diagnostic (size) criteria have been applied by pathologists examining specimens from human compared with rodents. Chemical agents with a wide variety of structures and pharmacological action have been noted as producing LCTs and it seems likely that an underlying hormonal disturbance (particularly of testosterone action or sustained elevated levels of luteinizing hormone) is responsible.

The chapter also considers the role that *in utero* exposure to agents might have on testicular organization and the subsequent induction of LCTs. The phthalate esters are good examples of environmental agents, which can produce a syndrome of reproductive tract abnormalities in male offspring following exposure of rats during pregnancy, including the production of dysgenetic areas in the testis that meet the size criteria for LCT diagnosis. The relationship of these events to the induction of peroxisome proliferator activated receptors (PPARs) is also discussed as a potential mode of action (as has been proposed for the liver tumors arising from long-term exposure to phthalates) with most evidence indicating that this does not seem to be a likely explanation for the induction of LCTs.

Key Words: Leydig cell tumor/Adenoma; mechanisms of tumor induction; phthalates; PPAR; testicular dysgenesis.

INTRODUCTION

Benign testicular Leydig cell tumors (LCTs) (adenoma) are relatively common neoplastic findings found during routine animal carcinogenic bioassays. They are frequently present in older animals at termination (2 yr) and are not normally found after 1 yr of treatment. Normally, these tumors are not noted as a cause of death and are present as circumscribed tumors within the testis, rarely becoming malignant, although, they can become very large and "burst" through the testicular tunica. Diagnosis in animal species is based purely on size and thus, there is a continuum between Leydig cell hyperplasia and adenoma. A large number of chemicals with widely differing structures have been shown to increase the appearance of LCTs when administered continuously in standard carcinogenicity bioassays. Although, these tumors have been noted in mice (predominantly after exposure to estrogenic agents), they are more frequently noted in rats. The range of different pharmacologies of agents that have resulted in increased incidence of LCTs, range from those with known hormonal activity, through agents that interact with orphan type receptors, to those that are known to be genotoxic (although, these are in the minority). Compound-induced LCTs are predominantly noted after exposure to agents, which do not directly interfere with DNA. In general, LCTs are rare tumors in human (~3% of all testicular tumors) and when noted are normally in response to an underlying hormonal disturbance (e.g., gynecomastia, altered serum hormone levels). Malignant LCTs are also rare findings in human (~10% of LCTs). Recent evidence has shown that Leydig cell hyperplastic nodules are relatively common findings in human testes at biopsy (and this would be from a highly selected population) for a variety of reproductive concerns. Morphological examination of these nodules would tend to support the notion that they are similar to the small LCTs commonly noted in rodents. Thus, the definition of what constitutes

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a Leydig cell tumor might indeed be different between veterinary and human clinical pathologists and it might be that the presence of small areas of hyperplasia (1–3 seminiferous tubules in diameter), which are not present with a major endocrinological profile might have a resemblance to the common LCTs noted in rodent bioassays. A critical question then arises as to whether environmental or pharmaceutical agents can produce or contribute to the findings of LC nodules in human? As such findings would not be easily detected in human (aside from biopsies for other reasons than cancer detection), and would not be associated with any major hormonal disturbance (as is the case in many rodent studies), this would be a difficult question to answer with any certainty. It certainly, would be prudent to consider that agents that could produce these tumors in rodents should be considered as potential risks for human and that they should not be dismissed based on their common occurrence in rodents versus their rarity as findings in human.

The advent of public and academic concern over agents, which have potential endocrine disrupting activity and contributions to human disease has also focused on the ability of these agents to induce testicular neoplasms. Such agents do elicit the induction of LCTs in chronic studies (using the standard rodent criteria), but interestingly, some attention is also being focused on the ability to induce a syndrome of responses, termed testicular dysgenesis, which might result from some insult occurring during fetal life affecting the normal development of the testis and male reproductive tract which has consequences for reproductive pathology in adult life. Indeed, recent rodent evidence has indicated that LCTs can be produced in young adults following short-term exposure to endocrine-active chemicals during critical developmental windows of sexual differentiation in gestation. Because *in utero* exposures and long-term follow up of the offspring are not normal components of rodent carcinogenicity bioassays (dosing usually, commences in young ~6–8-wk-old animals) there is no indication if this is a common, or rare, observation based on a specific pharmacological action. The focus of this short review will be to briefly review the biology of LCTs and the agents that can induce them in laboratory animals. The reader is referred to more comprehensive reviews of the multitude of agents, which can induce LCTs *(1)* for a more detailed exposition.

This short article will address two aspects that have not received significant previous attention, first, the induction of testicular lesions from *in utero* exposure that can produce Leydig cell neoplasia that could be classified as LCTs using standard criteria, and how recent research in these areas has impacted our views on the relevance of the rodent findings for humans.

Second, to briefly review the evidence of the role of peroxisome proliferator activated receptor (PPAR)-α (an important group of agents inducing these tumors are also PPAR-α ligands) in the etiology of LCTs.

BIOLOGY OF LCTs

The most frequently encountered testicular neoplasm of the mouse and rat is the LC adenoma. Incidence rates vary in different strains with the SD rat ranging from 1 to 5% and the F-344 rat reaching nearly, 100% *(2)*. Early neoplasms are common in 1 yr old F-344 rats and become increasingly more frequent with age *(3)*. Testicular neoplasia is less frequently observed in all strains of mice with incidence ranging from 1 to 2.5%. Tumors of the testis includes approx 1% of all human male neoplasms and in contrast to the rat, LCTs are rare in humans. LCTs in rodents generally occur in older animals, but in human can arise at any age, the majority between 20 and 60 yr *(4,5)*. The estimated incidence in man is 0.1–3 per million *(6)*. The proliferative lesions in LCs in rodents are similar and are observed as a continuous spectrum starting with smaller nodular foci of hyperplasia leading to large Leydig cell adenomas that can eventually replace the entire testis. The distinction between hyperplasia and adenoma is not always clear, with size being the major factor in the diagnostic criteria, with some debate over when focal hyperplasia becomes early neoplasia and there can be little morphological difference between a hyperplastic nodule and a small LC adenoma (*see* Fig. 1). Two arbitrary criteria have been developed for rodents. The National Toxicology Program *(7)* defined an adenoma as an aggregate of LCs, which is larger than one seminiferous tubule in diameter. The Society of Toxicologic Pathologists proposed that three seminiferous tubule diameters be set as the arbitrary separation of foci of hyperplasia from adenoma *(8)*. In contrast to rodents, no size criteria are used to distinguish between hyperplasia and adenomas in human. A distinct mass of LCs must be evident before it is considered a tumor *(9)* and human LCTs might be endocrinologically functional, producing androgens and/or estrogens *(4)*. A more recent article *(10)* has indicated that LC "micronodules" might be more common finding in human testicular biopsies than had been previously reported. As there are no stringently applied diagnostic criteria in humans, it might be that the incidence of LC adenoma has been under-reported in humans, as this recent article would

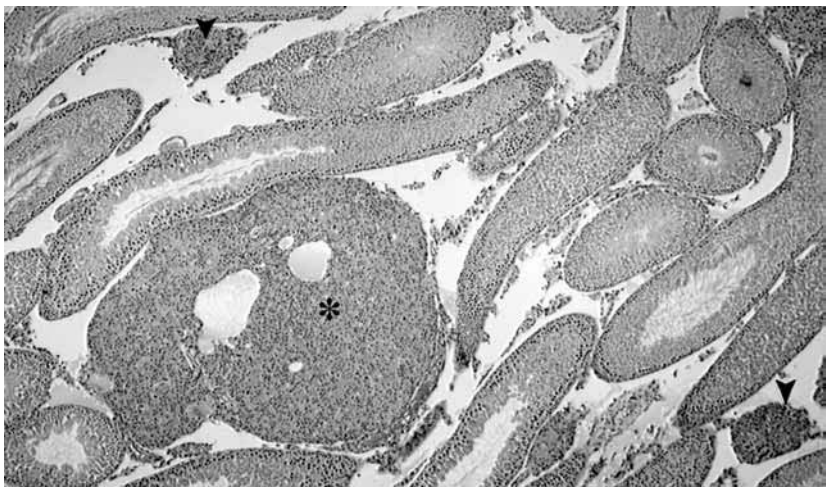


Fig. 1. Leydig cell hyperplastic nodules and adenoma. Photomicrograph of a classical Leydig cell adenoma (*) and Leydig cell hyperplastic nodules (▼) from a Sprague Dawley rat. Note that the diagnosis of adenoma is purely on size criteria (*see* "Biology of Leydig Cell Tumors" section). Micrograph from ref. *51*.

tend to indicate that some of these micronodules would be classified as adenomas if the rodent diagnostic size criteria had been applied. In both human and rodents malignant LCTs are rare. Criteria for malignancy being cellular anaplasia, increased and abnormal mitotic figures, invasion of the testicular tunica and epididymis, and rarely distant metastases *(9,11)*. The major difference between the testicular tumors observed in human and rodents (particularly the rat) are the high incidence of germ cell tumors in human and their occurrence in relatively young men (*see* "Testicular Dysgenesis and Phthalates" section). In rats, germ cell tumors are extremely rare, but LCTs can be almost 100% in incidence in certain strains (e.g., Fischer F-344) and occur most frequently in older animals.

POTENTIAL MECHANISMS FOR CHEMICAL INDUCTION OF LCTs

There are a number of potential mechanisms whereby, chemicals might induce LCTs primarily through a disruption in the Hypothalamic-Pituitary-Testis (HPT) axis. In most of these mechanisms, these agents will produce an elevation in circulating luteinizing hormone (LH) levels. Increases in LH have long been known to be able to produce LC hyperplasia and LCTs if the elevations are over a sustained period *(12,13)*. In studies with transgenic mice overexpressing hCG, it was noted that LCTs were induced in prepubertal mice and that these resembled functionally (through the use of phenotypic cell markers, e.g., thrombospondin-2) fetal type LCs rather than their adult counterparts *(14)*. Thus, agents can have a mode of action at different levels within the HPT axis in for example, in the central nervous system (CNS), hypothalamus, pituitary, and directly within the testis (*see* Fig. 2). Effects might be predominantly endocrine, paracrine, or autocrine (or potentially combinations of all).

Central Nervous System

Muselergine is an example of an agent that can act as a dopamine agonist in the CNS to affect prolactin action *(15)*, this decrease in prolactin has been proposed to downregulate LH receptors on LCs. The receptor downregulation results in decreased testosterone production and this results in a compensatory increase in circulating LH. The sustained elevation in LH has been proposed to result in LC hyperplasia and LCTs *(13)*. It is also possible that dopamine agonists (e.g., oxolinic acid *[16,17]*) can increase gonadotropin-releasing hormone (GnRH) levels, which subsequently increase LH levels resulting in the LC neoplasia.

Hypothalamus/Pituitary

Agents that directly block the androgen receptor (AR) like vinclozolin *(18,19)* or inhibit the metabolism of testosterone to the 5α-reduced androgen, and dihydrotestosterone (DHT) (e.g., finasteride *[20]*), could clearly produce LCTs by interference at the level of the hypothalamus and pituitary (and also potentially by blockade of the AR within the testis directly), preventing normal feedback of androgen to control GnRH and LH levels. This results in a sustained elevation in LH levels and the resulting LC hyperplasia and LCTs.

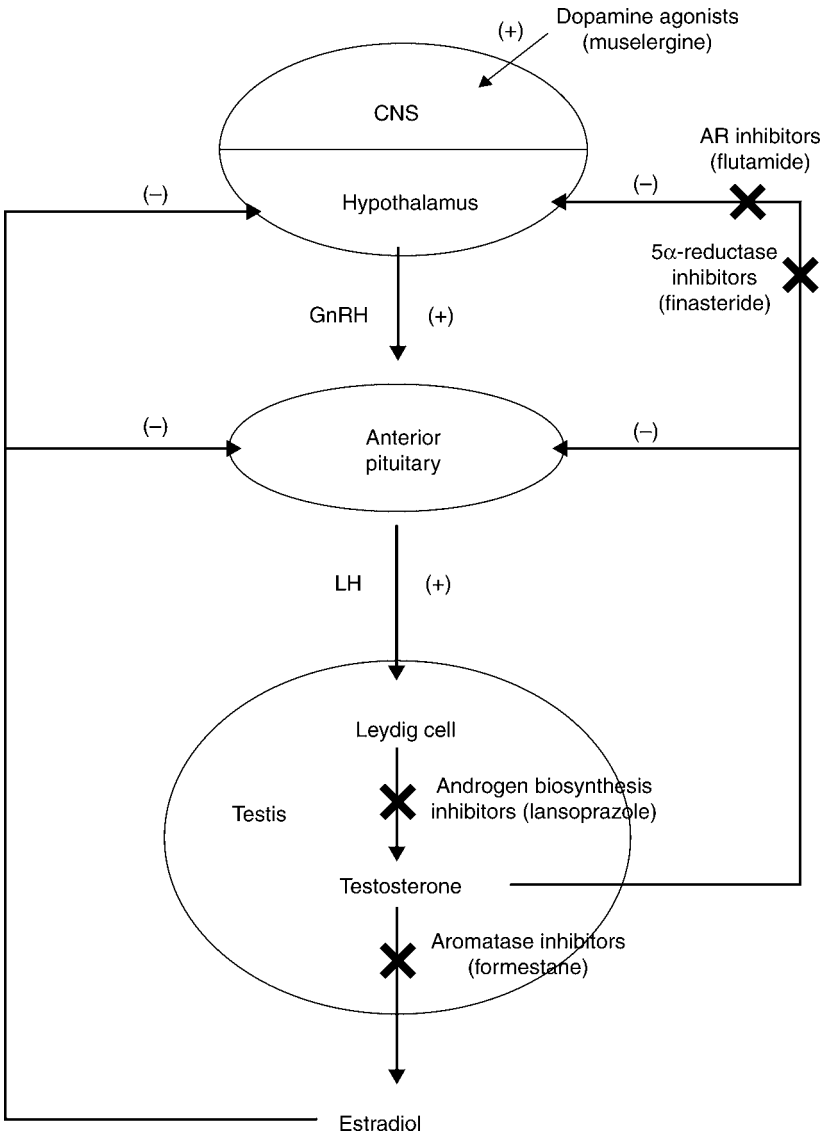


Fig. 2. Interference with HPG axis as potential modes of action for agents inducing rodent LCTs. Symbols: (+) feedback stimulation; (–) feedback inhibition; (✖) enzyme or receptor inhibition.

Testis

As androgens play a critical role in the feedback loop to maintain normal hormonal homeostasis, agents that might directly interfere with androgen biosynthesis (e.g., lansoprazole, *[21,22]* or potentially, aromatase activity (e.g., formestane *[23]*) to prevent the normal conversion of androgens to estrogens in LCs, might also interfere with circulating levels of LH. However, it is not unusual to note local changes in testosterone levels (which are significantly higher in testicular interstitial fluid than blood) without any sustained elevation of LH from a variety of different agents, which can induce LC hyperplasia and LCTs. In this instance, it would seem reasonable to propose that some local, paracrine mechanism is in place rather than the induction of these LC changes through the HPT axis. Testicular interstitial fluid contains numerous growth factors that could act as mitogens (e.g., insulin-like growth factor-1 (IGF-1), Platelet-Derived Growth Factor (PDGF) and cytokines that could alter Leydig cell function and induce growth *(24)*. Similarly, Leydig cell hyperplasia is frequently noted as a consequence of seminiferous tubular injury, suggesting an alteration in the paracrine control of normal LC function. No specific paracrine factor has been unequivocally linked to a chemical that can induce LCTs, but those examples of agents that can induce hyperplasia and tumors without any sustained increase in circulating LH, would be likely candidates to act through a disturbance in paracrine control.

Table 1
Examples of Chemicals With Different Pharmacology Know to Induce Leydig Cell Hyperplasia or Adenoma in Experimental Animals

Mode of action	*Species (strain)*	*Interstitial cell response*	*Examples*
Androgen receptor antagonists	Rat (Wistar, CD)	Adenoma	Biclutamide, flutamide, linuron, procymidone, vinclozolin
5α-reductase inhibitors	Rat (CD)	Hyperplasia	Finasteride
	Mouse (CD-1)	Adenoma	Finasteride
Androgen biosynthesis inhibitors	Rat (CD)	Adenoma	Lansoparazole, metronidazole, ethanol?
Aromatase inhibitors	Dog (beagle)	Hyperplasia	Formestane, letrozole
Dopamine agonists/ ↑Dopamine levels	Rat (Wistar and CD)	Adenoma	Mesulergine, norprolac, oxolinic acid
GnRH agonists	Rat (Wistar)	Adenoma	Histrelin, leuprolide, naferilin
Estrogen agonists/ antagonists	Mouse (BALB/c)	Adenoma	Diethylstilbestrol, estradiol, methoxychlor, tamoxifen
Calcium channel blockers	Rat (CD and Wistar)	Adenoma	Isradipine, lacidipine, nimodipine
Goitrogens	Rat (CD)	Hyperplasia	Propylthiouracil, ethylene thiourea
Peroxisome proliferators	Rat (CD and Wistar)	Adenoma	Diethylhexylphthalate, clofibrate, ammonium perflurooctanoate, gemfibrozil, perchloroethylene, trichloroethylene, methylclofenapate

From ref. *1*.

Any agent that could disrupt the feedback of testosterone or estradiol on circulating LH levels would also be potential candidate, as would agent that interferes with the liver metabolism of these steroids and thus alter their clearance and circulating levels. Although, no specific examples of such a mode of action have been reported, it has been suggested as part of the mode of action of lansoprazole *(25)*.

The large variety of agents known to produce LCTs indicate a wide variation in structure and potential modes of action, which are capable of eliciting LC hyperplasia and then in long-term (usually 2-yr rodent carcinogenicity) studies, LCTs. Table 1 illustrates the range of different pharmacologies that have been shown to produce LCTs in experimental animal species. The majority of agents when tested in conventional rodent bioassays in two species typically produce tumors in the rat and not the mouse. The most notable exceptions are the action of estrogenic agents (e.g., diethylstilbestrol and estradiol, *[26,27]*) that are regularly found to produce LCTs in the mouse, but usually not in the rat, reflective of the difference in the local effects of estrogens on LCs and the hormonal feedback pathways at the level of the hypothalamus and pituitary between rats and mice *(26,28)*. Interestingly, the overexpression of aromatase (the enzyme converting testosterone into estradiol) in genetically modified mice has been shown to induce LCTs in these animals that corresponded to more than doubling of serum estradiol levels *(29)*. Moreover, others have shown that this LCT induction can be prevented in the transgenic animals by administration of a known aromatase inhibitor, finrozole *(30)*.

The reader is also referred to a more comprehensive review of the agents and mechanisms that can induce LC hyperplasia and LCTs *(1)*.

TESTICULAR DYSGENESIS AND PHTHALATES

A testicular dysgenesis syndrome (i.e., a failure of normal *in utero* development of the testis) has been proposed to explain the secular increases in a number of human male reproductive deficits, including decreased semen parameters, increased incidence of cryptorchidism and hypospadias (two of the most common human birth defects), and increased incidence of testicular (germ cell-derived) cancer *(31–33)*. So far, no cause and effect relationship has been established between any environmental agent and these human deficits. However, the rodent data developed for phthalate esters following *in utero* exposure lends support to the hypothesis *(32)*.

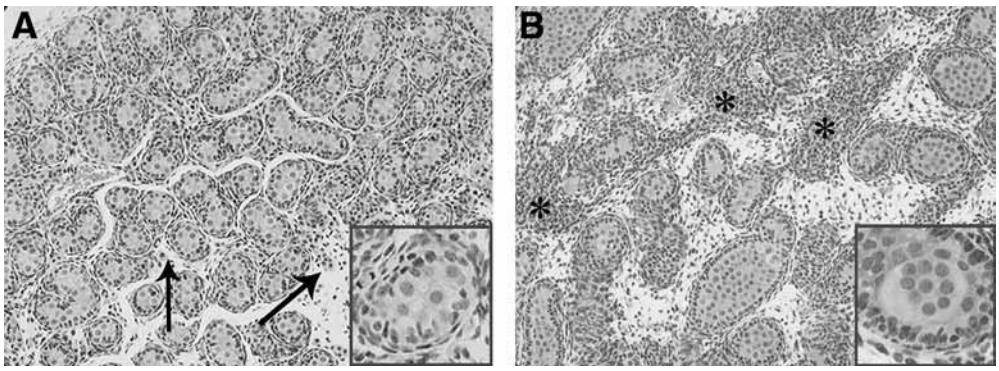


Fig. 3. Effects of *in utero* exposure to DBP on the induction of large fetal Leydig cell aggregates and multinucleate gonocytes. **(A)** Gestation day 21 rat testis from a control animal. Arrows indicate normal clusters of interstitial cells. Inset. Normal arrangement of gonocytes and Sertoli cells within the seminiferous cord. **(B)** Gestation day 21 testis from an animal treated *in utero* with 500 mg DBP/kg/d from gestation days 12–20. Note large aggregates of LCs (*) with Inset indicating multinucleate gonocytes compared with control. Micrographs from ref. *65*.

In the case of Dibutyl phthalate (DBP), *in utero* exposure alone has also resulted in testicular Leydig cell adenoma in 90 d-old offspring *(34)*. Usually, this tumor type is found at around 3–5% in control of the Sprague Dawley (CD) rat strain used in this study and generally is considered to be a tumor of old age (most frequently noted at the end of a 2-yr bioassay). A number of phthalates, including DBP, can produce a significant decrease in the testicular production of testosterone in vivo in rat fetal LCs *(35–37)* at time-points critical in male reproductive system development, which consequently results in failure of the Wolffian duct system to develop normally into the vas deferens, epididymis, and seminal vesicles *(38)*. Lower testosterone levels also impact the DHT-induced development of the prostate and external genitalia (testosterone is converted to DHT by 5α-reductase). DHT is also responsible for the normal apoptosis of nipple anlagen in male rats, resulting in the lack of nipple development, and also for the growth of the perineum to produce the normal male anogenital distance (approximately twice that of the female). Specific phthalates, including DBP, will produce malformations of the prostate, hypospadias, retained nipples, and a more female-like anogenital distance *(34,38)*. Testicular descent into the scrotum also, requires normal androgen levels and a failure of descent results in cryptorchidism. However, the initial stages of testicular descent involve the action of another Leydig cell product, insulin-like factor 3 (*insl3*), the message of which is also downregulated by di-(2-ethylhexyl) phthalate, (DEHP), DBP, and butyl benzyl phthalate *(39,40)*.

One of the earliest phthalate-related fetal effects observed in rats was disturbance of normal fetal testicular Leydig function and/or development *(35–37,41)*. This can result in fetal Leydig cell hyperplasia, or large aggregates of fetal LCs (at gestation day 21) in the developing testis (*see* Fig. 3). Down regulation of genes critical for normal steroid production and transport of critical intermediates is noted before observation of increased fetal Leydig cell aggregates *(37,41,42)*.

The decrease of *insl3* expression in rat fetal testes after DEHP (750 mg/kg/d), DBP (1 g/kg/d), or butyl benzyl phthalate (1 g/kg/d) exposure *in utero* on Gestation Day (GD) 14-18 *(39)* might be related to the increased incidence of cryptorchidism noted after fetal exposure to phthalates. Knockouts of this gene in mice show complete cryptorchidism *(43–45)*. The *insl3* gene is associated with Leydig cell differentiation and overall levels of testosterone, with high levels in fetal rat testes, which decrease after birth, rise again at puberty, but decrease again in old animals *(46)*. These authors also review the relationship of the SF-1 promotor found on the *insl3* gene with those found on many of the genes controlling the steroidogenic machinery in LCs. Although, human polymorphisms for *insl3* have not been reported, they have been noted for the *insl3* receptor (LGR8), which have been shown recently to be related to cryptorchidism in humans *(47)*.

The marked decrease in testosterone production by fetal LCs is critical to the subsequent reproductive tract malformations in rats exposed to specific phthalate esters *(35,36)*. This reduction in androgen leads to abnormal reproductive tract development *(38)*. For example, lower AR expression in epithelial cells of fetal epididymis is followed by Wolffian duct failure to coil and normally develop into the epididymis *(35,38)*. These androgen-dependent adverse outcomes affecting

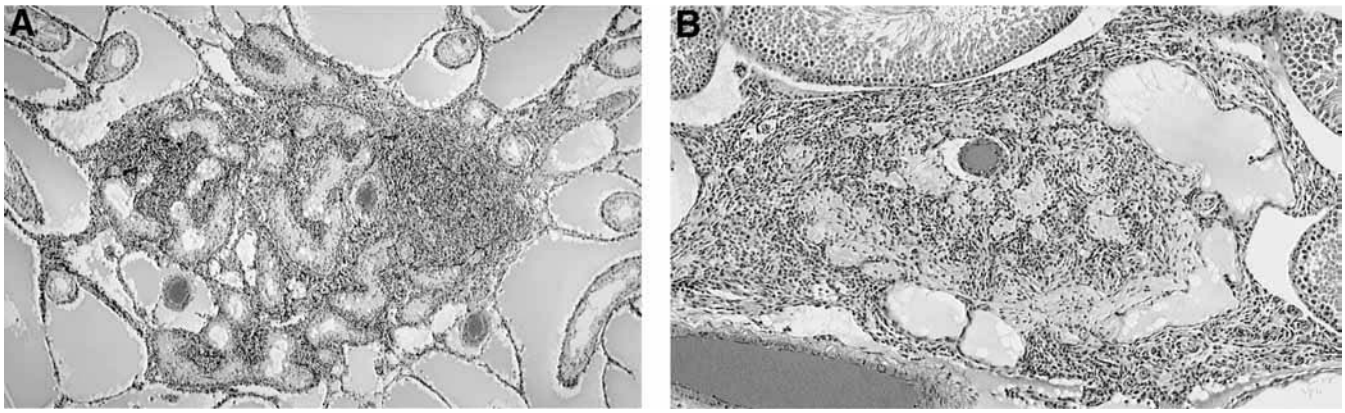


Fig. 4. Pictures of dysgenetic regions of testes from adult animals previously treated *in utero* with 500 mg DBP/kg/d. Note the wide variation in morphology between **A** and **B**, but both show regions of Leydig cell aggregation and contain tubule remnants that meet the overall size definition for a Leydig cell adenoma. **(A)** Area of testicular dysgenesis including convoluted, immature seminiferous tubules and large numbers of LCs. The surrounding tubules have marked seminiferous epithelial degeneration, and the interstitium is markedly expanded by interstitial fluid. **(B)** Higher magnification of dysgenetic area of the testis containing dilated tubules and numerous spindloid LCs. Micrographs from ref. *51*.

the Wolffian duct and prostate—together with hypospadias, cryptorchidism, retention of nipples and a permanently reduced anogenital distance—describe a syndrome of effects characteristic of specific phthalate esters *(48)*. This syndrome has parallels with the reported human testicular dysgenesis syndrome *(32,49)*, and also, shows similarities to other known human genetic syndromes involving impaired androgen responsiveness in sexual differentiation of the reproductive tract (for review, *see* ref. *50*).

The testicular tumors observed in rats following *in utero* exposure to DBP are Leydig cell adenomas that have a developmental origin *(34,42,51)*, and not the germ cell tumors most frequently noted in human. These dysgenetic areas meet the Society of Toxicological Pathologists' definition of a Leydig cell adenoma but clearly have a different morphology, normally are found in a distinct region of the testis (near the rete), and have a developmental origin (*see* Fig. 4). That is they do not increase in incidence in later life, but grow in size.

Abnormal fetal gonocyte development is hypothesized as a precursor event to germ cell cancer in humans *(52,53)*, and this has been seen in fetal testes from rats treated with DBP and DEHP *(36)* (*see* Fig. 3). Interestingly, small Leydig cell adenomas (using the rodent pathological definition) appear to be fairly common in humans, but have been termed micronodules, rather than tumors, according to a recent publication *(10)*. This is in stark contrast to previous articles where the presence of LCTs in humans is extremely rare (*see* above in Biology of LCT's). This difference in incidence is likely because of the use of different diagnostic criteria by toxicological vs human clinical pathologists, such that the presence of benign areas of LC proliferation (or potentially of a failure of apoptosis) that exceed three seminiferous tubules in diameter might be a more common observation in human than has previously been recognized, but has not been reported as a tumor.

The Role of PPAR-α

Peroxisome proliferators constitute a class of structurally diverse agents, which can induce rodent LCTs (*see* Table 1). These agents mediate their hepatic effects through the peroxisome proliferators activated receptor (PPAR)-α. These agents, in addition to producing LCTs in rodent carcinogen bioassays, also produce hepatic tumors and frequently pancreatic acinar tumors. It is not clear what role, if any, PPAR-α plays in the induction of the nonhepatic neoplasia, but it clearly has a crucial role in the induction of liver tumors *(54)*. DEHP is a good example of a PPAR-α agonist, which induces hepatic tumors in rodents and has also been found to induce LCTs after long-term administration in rats *(55)*. Studies with the PPAR-α-null mouse have shown that DEHP does not produce any of its hepatic effects in this knockout animal *(56)*; however, testicular effects (albeit later in onset) *(56)* and developmental effects *(57)* have been seen after DEHP administration in the null mouse. In other studies with potent peroxisome proliferators (e.g., WY-1463), it has been noted that no such peroxisome proliferation occurred in the testis, even though proliferation of these organelles was noted at comparable dose levels in the liver and LCTs were produced *(58)*. Moreover, the guinea pig is considered

a nonresponder to the hepatic effects of PPAR-α ligands, including DEHP *(59)*, but it is as equally sensitive as the rat to the induction of testicular toxicity *(60)*. The effects on reproductive development, also seem to rule out a role for PPAR-α. Rats appear to respond identically to the *in utero* exposure of DEHP and DBP (*see* in Testicular Dysgenesis, p. 388), yet DBP and its primary metabolite are very weak PPAR-α ligands (if at all) and certainly at the dose levels used to induce effects on testis development, there is no hepatomegaly in the offspring or dams. Therefore, it would be difficult to propose that two separate mechanisms would operate in the production of an identical syndrome of developmental reproductive changes. Moreover, a range of microarray and molecular studies (*see* for example ref. *61*) have shown that in the examination of the fetal testis, the phthalates that produce testicular effects (including DBP and DEHP) have a very similar profile of gene changes, which differs from those phthalates that do not induce testicular toxicity. In these studies, PPAR-α was not identified as a gene altered at the critical period of development for induction of the fetal testicular pathology and for the development of testicular dysgenesis and ensuing Leydig cell changes. The effects noted by DBP and DEHP on fetal testicular testosterone biosynthesis could imply that other PPARs could be involved (e.g., PPAR-γ), as ligands for these receptors can reduce cholesterol levels. PPAR-γ has been noted as showing increased expression (at the mRNA level) in the adult testis after acute, high dose DBP exposure *(62)*. However, studies with the potent PPAR-γ agonist, troglitazone, would suggest that although, lowered cholesterol does occur in animals after exposure to this drug, this does not appear to be through a PPAR-γ-mediated mechanism *(63)*.

CONCLUSIONS

A large variety of plausible mechanisms have been proposed for the induction of LCTs. Nearly, all have involved some alteration in the HPT axis resulting in an elevation in serum LH. For most agents, it would appear that this increase in LH levels needs to be for a sustained period of time, but this is not invariably the case. In some instances, it might be that the normal fall in LH noted in aged rats is not occurring at as fast a rate in the treated animals as in the corresponding controls. In other instances, only transient, if any, change is noted in serum LH, but increased incidences of LCTs are still found. However, testicular interstitial fluid contains numerous growth factors and other paracrine signals that are capable of affecting Leydig cell function (at least using in vitro systems). Thus, intratesticular control of growth (and potentially apoptosis) might be other important mechanisms that need to be further explored and studied. Studies of agents with hormonal activity (so called endocrine disruptors) have also indicated that differentiation and growth of LCs *in utero* might also be determinants of adult Leydig cell function, with examples of agents resulting in the presence of LCTs in young adult animals after short-term *in utero* exposure.

Thus far, no chemical has been shown unequivocally to produce LCTs in human. However, examination of potential mechanisms in experimental animals indicates that it is certainly possible that similar modes of action could exist if there was sufficient exposure. Thus, control of the HPT axis is very similar between rats and human and there is a potential for specific agents to affect circulating LH levels. Certain mechanisms noted in rats are unlikely to be relevant to human (e.g., dopamine and GnRH agonists) based on species differences in biology *(64)*. It seems likely that human and rat LCs might also have different sensitivity to LH based on Leydig cell receptor number and control of LH receptor expression (e.g., by prolactin in rodents and not humans) and that exogenous hCG will produce only LC hypertrophy in humans and not the LC hyperplasia seen in rats. It is also unlikely that human would ever be exposed to the dose levels known to be tumorigenic in rodents.

Other reviews have used the significant difference in incidence of LCTs between experimental rodents and human as another reason that LCTs in rodents are unlikely to be of relevance in predicting human risk. However, the appearance of Leydig cell micronodules as common findings in biopsies (at least in one report) and the differences in diagnostic criteria used for LCTs between rodents and human, might indicate that the small LCTs more commonly noted in control and treated rodents might indeed have a human counterpart. It is reasonable to suppose that small benign human LCTs might not be as rare as first believed, should similar pathological diagnostic criteria be applied. Whether these micronodules could be increased or caused by xenobiotic exposure would be speculation, but it would be prudent not to rule out agents that produce LCTs in conventional (and potentially *in utero* exposure) chronic studies as posing some risk to human in the overall assessment of chemical risk.

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28 Environmental Factors That Disrupt Leydig Cell Steroidogenesis

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SUMMARY

Leydig cells of the testis are responsible for the biosynthesis and secretion of androgens, critical for developmental and reproductive function in the male. Disruption of testosterone biosynthesis by environmental factors can cause sexual dysfunction, infertility, or sterility. The present review focuses on the toxicological effects of agricultural and industrial chemicals on Leydig cell steroidogenesis. An evaluation of the molecular targets of the reviewed chemicals identifies three major sites of action mediating their toxic effects on Leydig cell steroidogenesis. These are the luteinizing hormone-induced signal transduction pathway, cholesterol transport into mitochondria, and steroidogenic enzyme activity and/or expression.

Key Words: Agricultural and industrial chemicals; androgen; chemicals; cholesterol; cytochrome P450s; dioxins; enzymes; fertility; metals; pesticides; phthalates; PBR, StAR, steroidogenic enzymes; steroid dehydrogenases; testis; testosterone.

INTRODUCTION

It is well-established that androgens play critical roles in mammalian sex differentiation, exerting organizational effects on the morphogenesis of specific organs, and programming effects on neural functions and enzyme activities that are expressed later in life *(1,2)*. Clearly, the presence of adequate amount of androgens, at appropriate times, is a critical determinant of the male phenotype *(1–3)*. Consequently, reports of disruption of androgen production by environmental endocrine disruptors, chemicals that alter steroid biosynthesis and/or degradation, steroid binding to receptors, or transcriptional activation *(4–6)* are disturbing. Global decline in the numbers of spermatozoa produced by men, higher incidences of urogenital anomalies, such as cryptorchidism and hypospadias, poor semen quality, higher incidences of testicular cancer, and shorter pregnancy duration all have been attributed, at least in part, to environmental toxicants *(1,4,7–9)*. It has been postulated that environmental antiandrogenic compounds are likely to be involved in the etiology of these abnormalities, collectively termed testicular dysgenesis. Accordingly, the identification of chemicals in the environment that alter endocrine function, and the determination of the mechanisms, by which they function, have become high public and research priorities *(4)*. The effect of various drugs on Leydig cell androgen formation was previously reviewed *(10)*. The present review is focused on agricultural and industrial chemicals, which have antiandrogenic properties inhibiting Leydig cell steroidogenesis *(6,11–13)*.

STEROIDOGENESIS

Although, many tissues of the body have the ability to metabolize steroids, few tissues are able to synthesize steroid hormones from the substrate cholesterol. These tissues are the adrenals, gonads, placenta, and brain. Steroidogenesis in gonadal cells has been well-characterized and its regulation by peptide trophic hormones has been extensively studied *(14–16)*. In testicular Leydig cells, steroidogenesis is regulated by the trophic hormone and luteinizing hormone (LH). Binding of LH to the LH receptor induces the binding of the cytosolic domain of the receptor to a guanine nucleotide-binding (G) protein which subsequently stimulates adenylate cyclase activity, resulting in increased cyclic adenosine

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monophosphate (cAMP) formation *(17–20)*. cAMP stimulates the cAMP-dependent protein kinase, which phosphorylates serine and threonine residues on specific protein substrates *(21)*. Protein phosphorylation has been shown to be one of the regulatory steps in hormone-stimulated steroid formation *(15,22)*.

LH and cAMP regulate steroidogenesis in an acute (minutes) or chronic (hours) manner. The pool of cholesterol that is used for steroidogenesis consists of *de novo* synthesized cholesterol as well as cholesterol from peripheral sources taken up by the cells through the lipoprotein receptors *(23)*. The ability of steroidogenic cells to distinguish between cholesterol destined for membrane biogenesis and other functions that are common to all cells vs steroid biosynthesis, is not understood. Nonetheless, it has been well-established that the primary point of control of the acute stimulation of steroidogenesis by peptide hormones and cAMP involves the first step in this biosynthetic pathway, by which cholesterol is converted to pregnenolone. This is achieved by the C27 cholesterol side chain cleavage cytochrome P450 enzyme (CYP11A1) and auxiliary electron transferring proteins, localized at the inner mitochondrial membrane *(1–3)*. Pregnenolone then leaves the mitochondrion to undergo enzymatic transformation in the endoplasmic reticulum, which will give rise to the final steroid products. The rate-determining step in this pathway is not the enzymatic reaction by CYP11A1 but rather the transport of the precursor, cholesterol, from intracellular sources into mitochondria *(14,16)*.

Although, a number of molecules have been proposed to mediate this cholesterol transfer into mitochondria *(16,22)*, during the last 15 yr two cholesterol transport mechanisms, involving the peripheral-type benzodiazepine receptor (PBR) and the steroidogenic acute regulatory protein (StAR), have been identified *(24–27)*. PBR is a high affinity cholesterol binding protein, which is present at high levels in the outer mitochondrial membranes of steroid producing cells. PBR takes up free cholesterol from a cytosolic donor and transfers it from the outer to the inner mitochondrial membrane *(24,25)*. StAR is a 37-kDa protein, which contains a mitochondrial signal sequence and is able to induce cholesterol transfer across membranes; when in mitochondria StAR is cleaved to the 30-kDa mature protein *(26,27)*. In response to trophic hormones, StAR synthesis has been shown to parallel steroid synthesis *(16,26,27)*. Recent studies have reported that StAR need not enter mitochondria to stimulate steroidogenesis *(28,29)*, but rather might act through PBR at the outer mitochondrial membrane *(30)* to initiate the transfer to cholesterol from the outer to the inner mitochondrial membranes.

Following its production in the mitochondria, pregnenolone moves out of the mitochondria to the smooth endoplasmic reticulum, in which it is converted by the 3β-(HSD) hydroxysteroid dehydrogenase enzyme to progesterone *(31)*. Progesterone is then metabolized by 17α-hydroxylase/C17-20 lyase cytochrome P450 (CYP17) to produce 17α-hydroxy-progesterone and then to androstenedione. Androstenedione is converted to testosterone by 17β-HSD *(31)*. Androstenedione and testosterone can be further metabolized to estrone and estradiol, respectively, by cytochrome P450 aromatase (CYP19). Numerous studies have shown that the chronic effect of LH on steroidogenesis is because of the regulation of the expression of the messenger RNAs (mRNAs) for the steroidogenic enzymes by the trophic hormone *(15,31,32)*.

ENVIRONMENTAL AGENTS

More than 7×10^6 recognized chemicals are in existence and there are currently more than 80,000 chemicals registered with the Environmental Protection Agency (EPA). Chemicals are used in the creation and processing of thousands of products, including electronics, paper products, machinery, pharmaceuticals, personal care products, and household cleaners. Some chemicals are used to make plastic products, or in food processing and electricity generation. Three thousand of these chemicals are in heavy use, which means that more than 1×10^6 pounds are used in the United States per year *(33)*. Among the the high production chemicals, 43% have no toxicity data and only 7% have been completely screened to evaluate their impact on cell function, damage, carcinogenesis, and reproductive health *(33)*. Moreover, very little is known about their interaction with other chemicals and drugs. Indeed, if these chemicals were drugs, some of them are unlikely to have been approved for use. For this review, the chemicals have been divided into two large categories based on their use —agricultural and industrial. Exposure to some agricultural and industrial toxic agents either in the workplace or the home environment can cause sexual dysfunction, reproductive disorders, infertility, or sterility *(1,4–8,34–37)*.

AGRICULTURAL CHEMICALS

Organochlorines

Organochlorines are chemicals that contain carbon and chlorine atoms. Harmful organochlorines have

been detected in air, water, soil, sediment, fish, birds and human tissues, and fluids. These include:

1. Dioxin.
2. Industrial chemicals that are toxic in their own right, such as polychlorinated biphenyls (PCBs) and pentachlorophenol (PCP).
3. Chlorinated pesticides that are toxic, such as dieldrin and dichlorodiphenyl trichoroethane (DDT).

Organochlorines are stable, vapor forming, and can be carried by air currents. These chemicals can buildup in the food chain and thus, they can be found at high levels in species, at the top of the food chain, i.e., human, fish-eating birds, and marine mammals. Organochlorines concentrate in the fat and are slowly metabolized and excreted.

Coplanar PCBs, polychlorinated dibenzo-*p*-dioxins, and polychlorinated dibenzofurans are categorized as dioxins and related compounds. As they show adverse toxicities similar to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), acting through a common pathway through the aryl hydrocarbon receptor *(38,39)*. Aryl hydrocarbon receptor has been found in both rat and human Leydig cells *(40,41)*, suggesting that the various effects of these compounds on testicular steroid formation might be because of a direct effect on the Leydig cells.

PCBs have been widely used as pesticide extenders, in hydraulic fluids, rubber plasticizers, synthetic resin plasticizers, adhesives, wax extenders, cosmetics, varnishes, inks, electrical transformers, cutting oils, sealants, and caulking compounds. There are 209 PCB compounds called congeners. A congener might have between 1 and 10 chlorine atoms, located at various positions on the molecule. Many studies have examined the effects of PCBs on male reproductive function and reported numerous inhibitory effects *(42–45)*. These include in vitro and in vivo inhibition of androgen formation *(6,46–52)*. Detailed studies on the inhibitory effects of PCBs indicated that Aroclor 1248, a mixture of tritetra- and pentachloro congeners, inhibited 3β-HSD, CYP17, and 17β-HSD activities *(48)*. Treatment of adult male rats for 30 d with Aroclor 1254, resulted in reduction of circulating testosterone levels. Leydig cells isolated from Aroclor 1254-treated animals contained reduced numbers of LH receptors, and reduced CYP11A1, 3β-HSD, and 17β-HSD activities *(52)*. These detrimental effects of Aroclor 1254 could be partially prevented by coadministering vitamins C and E to the Aroclor 1254-treated rats *(52)*, suggesting that the effects seen were because of an alteration of the intracellular oxidant/antioxidant balance. Long-term exposure to pyralene, a PCB-based transformer oil, also, inhibited steroid formation in vitro by inhibiting 17β-HSD mRNA levels *(53)*.

Dioxin is the name for a class of organochlorines known as polychlorinated dibenzo-p-dioxins or dibenxofurans. The entire family consists of 75 different dioxins and more than 100 different furans. These compounds are byproducts of chemical industrial processes in cosmetic, pharmaceutical, and agricultural industries for the production, among other things of disinfectants and herbicides. The dioxin TCDD is the most toxic of the dioxins and furans. Toxicological studies have shown that TCDD reduces circulating testosterone levels in rats, and impairs spermatogenesis *(54–56)*. At least part of the effects of TCDD is because of the direct action of the compound on Leydig cells, where it affects both the volume of the endoplasmic reticulum and testosterone synthesis *(57,58)*. TCDD was later shown to inhibit cholesterol transport into mitochondria as well as CYP11A1 and CYP17 activities *(59–62)*. Treatment of wild-type mice with TCDD, reduced CYP11A1 and LH receptor expression *(63,64)*, whereas this effect was not seen in aryl hydrocarbon null mice *(63)*, demonstrating that indeed this receptor mediates the toxic effects of TCDD on Leydig cell steroidogenesis. The presence of aryl hydrocarbon receptor in human Leydig cells *(40,41)*, and the finding that, in men exposed to dioxin, there was a negative correlation between this chemical and testosterone levels *(65)*, suggests that exposed human are at risk because of the antiandrogenic effect of this chemical.

Today, aldrin is an organochlorine insecticide banned by the EPA. Aldrin accumulates in the testis, although, to a lesser extent than in other tissues *(66)*. Treatment of rats with aldrin inhibited intratesticular and circulating testosterone levels and affected spermatogenesis *(67)*. Heptachlor is an insecticide used in the control of termites and in the cotton industry. Its metabolite heptachlor epoxide, more toxic than the parent compound, has been detected in human testis *(68)*. Treatment of male rats for 2 wk with heptachlor suppressed circulating testosterone levels, whereas, LH and cortisol levels were increased *(69)*, suggesting that the compound might have a direct action on testicular steroidogenesis.

Chlorophenols have fungicidal and bactericidal properties. Two of the main members of this family are PCP and tetrachlorophenol. Both compounds were found in the human testis *(70)*, but no adverse effects on testicular function have been reported. Lindane, the γ-isomer of hexachlorocyclohexane, is one of the oldest pesticides. Hexachlorocyclohexanes were shown to

induce testicular dysfunction in rats and rams, which included reduction of androgen formation *(71–75)*. This effect was a result in part of decreased 3β-HSD and 17β-HSD activities *(71,76)*. Lindane was found in the human and rat testis *(71,77)*. When examined for its direct effects on Leydig cell steroidogenesis in vitro, it was shown to inhibit the hormone-induced steroid formation *(78,79)*. This effect was attributed to decreased numbers of LH receptors, reduced cAMP levels, and reduced cAMP-induced StAR protein levels *(79)*. The α- and δ-isomers of hexachlorocyclohexane also demonstrated similar inhibitory effects on steroidogenesis *(78)*.

DDT was identified as a long-lasting insecticide against the clothes moth. It was extremely effective against flies and mosquitoes and was used extensively as a domestic and agricultural pesticide until the EPA banned it because of its long residual life and its accumulation in the food chain. The terms DDT or DDTs are often used to refer to a family of isomers (p,p′-DDT and o,p′-DDT) and their breakdown products (p,p′-dichlorodiphenyldichloroethylene [DDE], o,p′-DDE, p,p′-dichlorodiphenyldichloroethane [DDD], and o,p′-DDD). DDT and its metabolites were shown to exert gonadal toxicity and to affect sexual development in the male rat and other species *(80–84)*. Its effects included reduction of circulating testosterone levels *(85–87)*. In human, o,p′-DDD (mitotane) has been used to treat adrenocortical carcinoma, associated with high steroid production *(88,89)*. Patients treated with the compound had reduced levels of all circulating steroids measured, including testosterone *(88)*, suggesting an effect on CYP11A1 and 3β-HSD *(88)*. In a case report, treatment with o,p′-DDD resulted in primary hypogonadism, reversed a few months after the end of the treatment *(89)*.

In search of the mechanism of action of DDT, it is important to recognize that o,p′-DDT, but not its metabolite o,p′-DDE, is an environmental estrogen *(90)*, and therefore, some of its effects on steroidogenesis might be because of this property of the compound. In contrast, the metabolite p,p′-DDE binds to the androgen receptor and inhibits androgen-induced gene transcription *(6,90,91)*.

Although, DDT was banned, its analog 2,2-*bis*(p-hydroxyphenyl)-1,1,1-trichloroethane (methoxychlor) remains in use as an insecticide. Methoxychlor attaches to soil particles, does not dissolve easily in water or evaporate easily into the air, and is long-lasting. Methoxychlor is converted to the bioactive metabolite 2,2-*bis*(p-hydroxyphenyl)-1,1,1-trichloroethane. Methoxychlor was found to induce testicular toxicity *(92–94)*, including reduction in hormone-stimulated testosterone production *(94,95)*, without affecting serum LH levels *(95)*. 2,2-*bis*(p-hydroxyphenyl)-1,1,1-trichloroethane was found to inhibit in vitro the basal and LH-stimulated testosterone production *(96–98)*, and the effects were localized at CYP11A1 expression and activity *(96,97)*. The site of action of methoxychlor at CYP11A1 was further confirmed in ex vivo studies *(95)*. Interestingly, although, methoxychlor and its metabolite are considered to have estrogenic activities, their effects on CYP11A1 do not seem to be mediated by the estrogen receptor *(97)*. Originally, tris(4-chlorophenyl)methanol (dicofol) was manufactured from DDT, and is a long-lasting miticide used on a wide variety of fruits, vegetables, and ornamental and field crops. Although, dicofol had no effect on testicular morphology and serum testosterone levels in in vivo studies in the rat *(99)*, it was recently shown to inhibit 17β-HSD in carp testicular microsomes *(100)*.

Organophosphates

Organophosphates is a generic term to include all the insecticides containing phosphorus. They exert their toxic action by inhibiting the cholinesterase enzymes of the nervous system, which results in the accumulation of acetylcholine. Some of the aliphatic organophosphates were tested for their effect on androgen synthesis. Dimethoate was shown to reduce testosterone formation by the testis in vivo *(101)*. In search of the mechanism of action of dimethoate, Walsh et al. *(102)* demonstrated that it acts by inhibiting the transcription of the StAR gene, thus, blocking the transport of cholesterol into mitochondria and subsequent androgen formation. Dimethoate was also found to inhibit CYP11A1 activity *(102)*. Treatment of male rats with other organophospates, such as malathion and dichlorvos, had no effect on androgen production by the testis *(103,104)*.

Among the organophosphates, the effects of the herbicide glyphosate (roundup) on steroidogenesis was studied in detail. Roundup was initially shown to inhibit the cAMP-induced steroid synthesis by MA-10 Leydig cells in culture without causing any toxicity *(105)*. This effect was attributed to an effect on the mature (30-kDa) StAR expression *(105)*. However, further studies demonstrated that the effect on StAR protein expression was not because of the active compound glyphosate but to the surfactants used as inactive ingredients in roundup *(106)*. Indeed, several surfactants were tested for the effect on MA-10 Leydig cell steroidogenesis, and each produced a concentration-dependent decrease in progesterone synthesis. Decreased progesterone synthesis was coincident with surfactant-induced mitochondrial membrane damage and decreased

levels of the 30-kDa form of the StAR protein. However, there was not a parallel decrease in levels of the 37-kDa form of the StAR protein. Transient transfection experiments with a plasmid coding for the StAR protein in control cells and treated with a surfactant demonstrated no effect on levels of the 37-kDa form of the protein. Therefore, the dose-dependent decrease in StAR protein levels in MA-10 cells treated with surfactant appears to result from increased degradation of the 37-kDa form of the StAR protein rather than from an effect on StAR protein expression *(106)*. The results of this study underscore how an effect on the integrity of the mitochondrial membrane can affect StAR-mediated progesterone synthesis in human chorionic gonadotrophin (hCG)-stimulated MA-10 Leydig cells and the nonspecific action of a variety of surfactants on cellular function in an in vitro test system.

1,2-Dibromo-3-Chloropropane

1,2-Dibromo-3-chloropropane (DBCP) is a testicular toxicant in various species, which affects mainly the seminiferous epithelium and spermatogenesis *(107–109)*. The route of exposure seems to be a critical factor for the testicular toxicity of DBCP *(107–113)*. Although, many studies concluded that Leydig cells in the testis are secondary targets for DBCP toxicity, Kelce et al. *(114)* demonstrated that DBCP has a direct effect on CYP17 activity, when administered as a challenge to rats pretreated with 2-hexanone. Analysis of gonadal function in human exposed to DBCP indicated oligospermia and hypogonadism, which is reversed with time *(115–117)*.

Carbamates

Carbamates, derivatives of carbamic acid, are used as insecticides. They also inhibit the enzyme cholinesterase. Chronic exposure of rats to the carbamate methomyl, resulted in reduced circulating testosterone levels, whereas, LH levels were increased *(118)*. The thiocarbate herbicide molinate also caused testicular damage in rats *(119,120)* and it was shown that this chemical specifically concentrates in Leydig cells of the testis *(120)*. Treatment of rats with molinate resulted in decreased circulating and intratesticular testosterone levels.

Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are a family of about 100 chemical compounds with a molecular structure consisting of at least two fused aromatic rings. PAHs can enter the aquatic environment directly through industrial and municipal effluents, oil spills, and PAH emissions, including those produced by the use of tobacco products. Leydig cells were first identified as the site of binding and metabolism of the PAH 7,12-dimethylbenz[a]anthracene (DMBA) in the testis *(121)*. DMBA metabolites subsequently, inhibit DNA replication in the seminiferous epithelium *(122)*. Exposure of MA-10 and rat Leydig cells to DMBA resulted in the inhibition of the cAMP-stimulated steroid formation *(123)*.

Fungicides

Most fungicides inhibit steroid synthesis by acting at cytochrome P450 enzymes. Imidazoles and the structurally related N-substituted triazoles are potent antifungal compounds used in human, birds, and plants *(124)* because of their inhibitory effect on the fungal sterol 14α-demethylase cytochrome P450. Imidazoles, have been shown to affect testicular function and male reproduction *(125–127)*. Ketoconazole, an orally administered broad spectrum antifungal drug representative of the imidazole family, reduced serum and saliva testosterone, but not LH levels in human volunteers *(128–130)*. The concentrations of ketoconazole used were close to those used in therapy. A similar effect of ketoconazole in rats was shown as a result of the inhibition of 3β-HSD, CYP17, 17HSD, and CYP19 enzymes *(131)*, although, the major effect was at CYP17 *(132)*. Ketoconazole inhibited both basal and hCG-stimulated testosterone production in isolated Leydig cells in vitro *(128,130)*. This inhibitory effect of ketoconazole was observed in human, rodents, dogs, pigs, and stallions *(132–134)*, although, ketoconazole was more potent on human interstitial cells *(134)*.

More recently, ketoconazole was also shown to inhibit adenylate cyclase in MA-10 mouse tumor Leydig cells *(135)*. Isoconazole, miconazole, econazole, and clotrimazole exerted similar effects on Leydig cell steroid synthesis, although, metronidazole and levamisole were ineffective *(136)*. In detailed studies, Walsh et al. *(137)* demonstrated thatmiconazole and econazole reduced the hCG-stimulated cholesterol transport in addition to their effect on CYP17. This effect was because of an effect of the compounds on cAMP-induced StAR expression *(137)*. More recently developed fluconazole, a *bis*-triazole, did not affect serum testosterone levels in human volunteers and at very high concentrations showed a weak inhibition of androgen production, in rat Leydig cells in vitro *(137)*. However, 1-mo treatment of rabbits with fluconazole resulted in reduced circulating testosterone levels and affected fertility, although, in a reversible manner *(138)*. The fungicide fenarimol was

found to induce infertility in the male rat *(139)* and it has been shown to directly inhibit CYP19 *(140)*, although, the impact of this effect on androgen formation has not been studied.

Trialkyl Organotins

The tributyltin compounds, a subgroup of the trialkyl organotins, are active ingredients in biocides used to control a broad spectrum of organisms (pesticides, microbicides, and fungicides). Organotins, including tributyltin, affected spermatogenesis in various species *(141,142)*. In vivo generational studies indicated that tributyltin did not affect serum testosterone and LH levels, but inhibited CYP19 activity *(143)*. In vitro studies demonstrated that tributyltin and triphenyltin inhibited 3β-HSD, CYP17, 17β-HSD, and CYP19 enzyme activities *(144–147)*.

Triazines

The herbicide 2-chloro-4-ethylamino-6-isopropylamino-s-triazine (atrazine) is the most widely used member of this family of compounds. There are numerous reports of atrazine exposure having significant effects in nonmammalian species *(148–150)*. Among these findings, exposure of larvae of male *Xenopus laevis* to atrazine was shown to induce hermaphroditism and demasculinization, and lead to reduced plasma levels of testosterone in sexually mature males. Exposure of adult male rats to atrazine has been reported to result in reduced serum and intratesticular testosterone concentrations and to affect the growth of androgen-dependent organs *(151–155)*. Recently, it was shown that atrazine administered during the peripubertal period resulted in reduced serum LH and testosterone concentrations, intratesticular testosterone levels, seminal vesicle weight, ventral prostate weight, and intratesticular testosterone concentration *(156)*. These data suggest that atrazine affects the production of testosterone by Leydig cells, and/or that it alters testosterone metabolism within the testis.

2,4-Dichlorophenoxyacetic Acid

2,4-Dichlorophenoxyacetic acid (2,4-D) is a widely used herbicide, which belongs to the phenoxy class of chemicals. Chronic treatment of rats with 2,4-D resulted in morphological changes in Leydig cells, germ cell depletion, and sterility *(157,158)*. 2,4-D has been shown to inhibit the hormone-induced testosterone synthesis by isolated rat Leydig cells in vitro *(159)*. No detailed studies on its mechanism of action have been undertaken, although, considering that more than 40×10^6 pounds of 2,4-D are used annually in the US *(EPA)* these studies are warranted. It is important to note that in addition to p,p′-DDT and p,p′-DDE, the pesticides vinclozolin, procymidone, linuron, iprodione, chlozolinate, p,p′-DDT, and p,p′-DDE exert antiandrogenic properties affecting sex differentiation by acting at the androgen receptor level to inhibit androgen-induced gene expression. A number of reviews have been devoted to the antiandrogenic properties of these compounds *(6,160,161)*. In addition, some chemicals such as o,p′-DDT and certain PCBs have estrogenic properties. It is possible that some of the effects of these compounds on androgen formation are mediated by the androgen or estrogen receptor.

Industrial Chemicals

Monomers and Plasticizers

Bisphenol A

Bisphenol A is a monomer used in the production of epoxy resins and polycarbonate plastics. The resins are used to coat metal products, such as food cans, bottle tops, and water supply pipes and the plastics are used in food and drink packaging applications. Bisphenol A is also among the polymers used in dental treatment. Bisphenol A binds to both estrogen receptor-β *(162,163)* and the androgen receptors *(163)*, and thus, has been tested for its effects on male reproductive development. The results obtained indicate minor effects on spermatogenic function and fertility *(164–166)*, and on testosterone formation both in vivo *(167)* and in vitro *(167,168)*. In vitro studies of isolated rat Leydig cells demonstrated that its effects were because of decreased expression of CYP17 *(166)*. In contrast to these findings, experiments using the mLTC-1 Leydig cell line indicated that bisphenol A inhibits cAMP and steroid synthesis by blocking the LH receptor–adenylate cyclase coupling *(169)*.

Phthalic Acid Esters

Among the environmental agents affecting male reproductive function, phthalates might be the most well-studied. For that reason, these studies are presented in more detail, but the reader is also referred to Chapter 27 by Paul Foster in this volume. The phthalic acid esters (PAEs), or phthalates, are used as plasticizers in manufacturing a variety of plastics, consisting up to 40% of the volume of the plastic *(170)*. Worldwide, manufacturers produce an estimated one billion pounds of PAEs per year *(171)*. As PAEs are loosely held between the interstices of the polymer matrix, they are capable of leaching out of the plastic. Human and animals might be exposed to PAEs by ingestion, blood and food storage containers, the water supply, or skin

contact (PAEs are constituents of some perfumes, cosmetics, and pesticides). PAEs have been shown to produce acute toxicity in rodents *(172–175)*, affect fertility and litter size *(176–178)*, cause detachment of germ cells from the seminiferous tubular epithelium *(179–184)*, and affect Sertoli cell function *(185–188)*. Recent studies have shown that PAEs influence steroid synthesis *(189)*. In the mouse, for example, di-(2-ethylhexyl) phthalate (DEHP) *(190)* and monoethylhexyl phthalate (MEHP) *(191)* administration, resulted in decreases in testicular testosterone; and Jones et al *(192)* showed that MEHP inhibited the LH-stimulated testosterone secretion in primary cultures of rat Leydig cells. These data, taken together with the observation that coadministration of testosterone and DEHP prevented DEHP-induced testicular toxicity *(193)*, indicated that the Leydig cell might be a target for PAEs. Consistent with this, it was recently reported that MEHP inhibited hormone-stimulated steroid synthesis in a dose-dependent manner, in both purified adult rat and mouse tumor Leydig cells *(194,195)*. Importantly, micromolar concentrations of MEHP were used in the studies, concentrations that are considered to be physiologically relevant because micromolar concentrations of MEHP have been found in human blood, urine, and semen *(196–200)*.

Recent studies have indicated that the male reproductive system is particularly sensitive to PAEs when exposure occurs early in development, and that PAEs can affect the developing male reproductive system. First, following its administration to pregnant mothers, DEHP was found in maternal blood, amniotic fluid, placenta, and fetal tissue, indicating that it crosses the placenta and thus, might have a direct effect on developing embryonic tissue *(201)*. Second, Imajima et al. *(202)* reported that prenatal exposure to PAEs caused cryptorchidism postnatally, and suggested that androgen inhibition during a brief period of embryonic development was responsible *(203)*. This hypothesis was supported by studies showing that the reproductive tract malformations induced by dibutyl phthalate (DBP) and DEHP in androgen-dependent tissues, in male rat offspring, were similar to the effects produced by the antiandrogens vinclozolin, procymidone, and flutamide *(6,11,12)*. Thus, it is postulated that DBP and DEHP alter fetal development through an antiandrogenic mechanism.

There is agreement that PAE is able to inhibit of fetal Leydig cell androgen production *(12,13,204–210)*. There is also an evidence that exposure of the fetus to PAE might induce permanent imprinting, later resulting in demasculinization *(211)*. Consistent with this, a recent study indicated that treatment of pregnant dams with DEHP or DBP led to decreased serum testosterone levels in male offspring at 21 or 35 d of age *(208,212)*. However, this effect was no longer evident at 90 d of age *(212)*. The recent results *(213)* show that administration of increasing doses of DEHP during gestation resulted in significant dose-dependent reductions in serum testosterone and estradiol concentrations in 60 d-old rats. It should be noted that although there are numerous phthalates the two most abundant *(214)* and well-studied are DBP and DEHP. In search of the mechanism of action of phthalates on Leydig cell steroidogenesis, PBR was identified as one of the target proteins affected. It was demonstrated that the bioactive metabolite MEHP exhibits antiandrogenic properties through a mechanism, which involves inhibition of hormone-induced cholesterol transport into mitochondria, the rate-determining step in steroid biosynthesis, followed by inhibition of steroidogenesis *(194,195)*. Analyses of in vitro and in vivo data indicated that this effect is mediated, at least in part, by transcriptional suppression of the Leydig cell PBR gene *(195)*. Phthalates are members of the peroxisome proliferators class of compounds acting through the peroxisome proliferator-activated receptors (PPARs), a family of receptors, which belong to the nuclear hormone receptor gene superfamily *(215,216)* and mediate their effects in cells. Peroxisome proliferators are a large class of structurally diverse industrial and pharmaceutical chemicals, such as the fibrate hypolipidemic drugs bezafibrate and clofibrate, prescribed for prevention of coronary heart disease, herbicides, perfluorodecanoic acid (PFDA), and PAEs *(215,216)*. Leydig cells have been found to express the PPARα and PPARβ/δ proteins *(195,217,218)*. In vivo acute treatment of adult mice with peroxisome proliferators, including DEHP, was shown to decrease both testis PBR mRNA and circulating testosterone levels *(195)*. In parallel studies, the MEHP-induced reduction of cultured Leydig cell PBR mRNA levels was found as a result of a direct transcriptional inhibition of the PBR gene *(195)*.

The gross similarity of the effects seen in animals treated with either PAEs or classical antiandrogens (androgen receptor antagonists), such as flutamide *(219)*, vinclozolin *(220)*, procymidone *(221)*, and linuron *(222)*, imply that PAEs, such as DBP and DEHP act as antiandrogens. The following observations indicate that PAEs does not act as androgen receptor antagonists, but rather that their antiandrogenic properties result from their inhibition of fetal Leydig cell androgen synthesis:

1. Parks et al. *(204)* and Foster et al. *(205,206,210)* demonstrated that DEHP and DBP inhibit fetal rat testosterone production during sexual differentiation,

an effect that is not caused by the androgen receptor antagonists flutamide *(206)* and linuron *(222,223)*.
2. DEHP and DBP as well as their monoester metabolites, MEHP and MBP, do not act as androgen receptor ligands in vitro *(204–206)*.
3. DEHP, administered at a dose that produces hypospadias, is more potent than the androgen receptor antagonists *(204,211)*.
4. DBP, unlike the androgen receptor antagonist flutamide, induces regions of Leydig cell hyperplasia and gonocyte degeneration in the fetal testis *(13)*.

DEHP might also inhibit androgen formation by increasing androgen metabolism to estrogen *(224)*. Indeed, prolonged exposures of immature rats to DEHP were shown to result in increased expression of the cytochrome P450 aromatase, the enzyme responsible for the metabolism of androgen to estrogen *(224)* and present in Leydig and Sertoli cells of both fetus and adult *(225–227)*. Whether this is reflected by increased estrogen formation in the blood and whether this holds true for adult rats exposed *in utero* to DHEP is not known. However, it is known that DEHP has no estrogenic activity *(228)*. In support of the proposed studies, it was recently shown that a high-dose exposure of pregnant rats to DBP reduced cholesterol transport to mitochondria, decreased the expression of proteins involved in fetal androgen formation *(229)*, suggesting that fetal Leydig cell steroidogenesis is a target mechanism for DBP. Detailed studies by Gaido and colleagues *(207,230–233)* identified among other genes StAR, PBR, CYP11A1, 3β-HSD, CYP17 as target genes for phthalate in the fetal Leydig cells.

Perfluorinated Compounds

Perfluorinated compounds are persistent and bioaccumulative chemicals with a broad range of industrial applications. These compounds are used in industry as lubricants, surfactants, plasticizers, wetting agents, and corrosion inhibitors. Perfluorinated compounds belong to the class of the earlier-described peroxisome proliferators *(215,216,234,235)*. The perfluorinated carboxylic acid PFDA was reported to induce early degenerative changes that progressed to tubular atrophy *(234,235)*. The same peroxisome proliferator was also reported to suppress plasma testosterone levels, without affecting plasma LH concentrations, thus, inducing a decrease in accessory organ weights *(236)*. As implants of testosterone reversed the effect of PFDA, and testes from PFDA-treated rats were lesser responsive to LH than control testes, it was concluded that this chemical exerts a direct effect on the hormone-stimulated testosterone biosynthesis *(236)*. Indeed, PFDA inhibited in a time- and dose-dependent manner the hCG-stimulated MA-10 mouse tumor and rat Leydig cell steroidogenesis *(237)*. This effect was localized at the level of cholesterol transport into the mitochondria and PFDA was found to inhibit PBR mRNA levels *(237)*. Furthermore studies indicated that PFDA accelerated PBR mRNA decay, suggesting that PFDA inhibits the Leydig cell steroidogenesis by affecting PBR mRNA stability.

Perfluoro-octanoates (PFOAs) also exert testicular toxicity ranging from Leydig cell tumor formation for ammonium PFOA *(238)* to impairment of sperm production and maturation for PFOA sulfonate *(239)*. PFOA was shown to reduce circulating and interstitial fluid testosterone levels in the rat with increased circulating estradiol levels, indicating a direct effect on CYP19 *(240)*. PFOA exerted a direct effect on Leydig cells as it inhibited in a dose-dependent manner the hormone-stimulated steroid formation by isolated rat Leydig cells *(159,240)*. Interestingly, Leydig cells isolated from animals treated with PFOA maintained their ability to synthesize androgen, suggesting that the effect of PFOA on steroidogenesis is reversible *(240)*.

Metals

Arsenic

Arsenic is used in the production of herbicides, insecticides, and rodenticides and has been identified as a frequent pollutant in the air, soil, and water, because of its widespread use in agriculture and industrial procedures, such as mine tailings and smelter wastes. Studies have suggested that it might be toxic for fish and wildlife. The effects of chronic exposure to arsenic on reproductive functions were examined by exposing mice for 1 yr to sodium arsenite added in the drinking water at a dose that human are likely to encounter through drinking water. Arsenic exposure was found to decrease testicular, but not epididymal and accessory sex organ weights. The activities of several testicular enzymes, including 17β-HSD and the sorbitol dehydrogenase and acid phosphatase were significantly decreased, suggesting an effect on Leydig cell functions. Moreover, increased occurrence of abnormal sperm and a decrease in sperm count and sperm motility were observed in arsenite-exposed mice.

In view of these results, the authors concluded that arsenic exposure at levels that can be found in drinking water of human populations might exert toxic effects on spermatogenesis and testicular steroidogenesis *(241)*. Reproductive toxic effects of arsenic were also observed in adult rats treated with either arsenite administered either ip or through drinking water for a long period. These effects included dose-dependent

decreases in testes, seminal vesicles, and ventral prostate weights, epididymal sperm count, and plasma concentrations of LH, follicular stimulating hormone (FSH), and testosterone *(241–243)*. Sodium arsenite delivered through drinking water to adult rats for up to 4 wk, induced germ cell degeneration together with decreases in plasma LH and FSH, plasma and testicular testosterone concentrations, and 3β-HSD and 17β-HSD activities *(243)*. Because coadministration of hCG with sodium arsenite was found to partially prevent the deleterious effects of arsenite whereas estradiol was found to enhance the effects of arsenite, the authors proposed that the toxic effects of arsenic on testis might involve pituitary gonadotrophins and that it might act through an estrogenic mode of action *(243)*. In summary, these studies indicated a toxicity of arsenic on Leydig cell steroidogenesis in part because of a decrease in 3β-HSD and 17β-HSD activities, whereas the decrease in LH and FSH observed in these studies suggested that the effect on Leydig cells might be indirect.

Cadmium

Cadmium is another heavy metal encountered in the environment in relation to industrial activities including smelting, oil and coal burning, and from the wear of tires. Diverse toxic effects of cadmium have been described in birds. Cadmium is also known to affect testicular function in rodents and cadmium exposure has been shown to induce testicular degeneration. A single dose of cadmium was found to induce the formation of testicular Leydig cell tumors at a rate higher than in normal conditions. Whereas, testosterone implanted in the rats was shown to abolish the occurrence of both cadmium-induced and spontaneously occurring Leydig cell tumors, but could not protect the testes from chronic testicular degeneration. Thus, cadmium-induced hypofunction of the testes appeared to be a consequence of the decrease in circulating testosterone, whereas the induction of Leydig cell tumors had a different cause *(244)*. A direct effect of cadmium toxicity on testis was further demonstrated using isolated rat Leydig cells exposed to several doses of cadmium chloride. The results showed that Leydig cell viability and basal and hCG-induced testosterone production were decreased by cadmium exposure compared with controls *(245)*. In the same study, cadmium increased DNA damage, indicating that cadmium exerted direct cytotoxic and genotoxic effects on Leydig cells. In search of a mechanism mediating the deleterious effects of cadmium in the testis, investigators recently showed that the injection of a single dose of cadmium in rat caused an increase in testicular reactive oxygen when measured two days after treatment. There was a decrease in several parameters of Leydig cell function, including StAR mRNA, 3β-HSD and 17β-HSD activities, and serum testosterone levels, suggesting that cadmium acted by inducing a ROS-mediated inhibition of testicular steroidogenesis *(246)*. This hypothesis was supported by data showing that normal testicular function could be restored by treating cadmium-exposed rats with vitamin C or E supplements *(246)*.

Chromium

Environmental exposure to chromium occurs through contamination of sewage and solid wastes and pollution of drinking water near industrial sites, such as tanneries, welding, and chrome-plating industries. At high concentrations, chromium is a mutagen and a carcinogen *(247)*. Long-term in vivo exposure of rats to hexavalent chromium (CrVI) was found to induce a decline in the Leydig cell population and a significant inhibition of 3β-HSD, as well as a decrease in serum testosterone *(248)*. An in vivo study performed on rabbits, also, reported a decrease in testosterone levels, and body, testis, and epididymis weights following exposure to CrVI *(249)*. However, in a study comparing the effects of several heavy metals on isolated rat Leydig cells, chromium was shown to have no deleterious effect on either viability or hormone-induced steroidogenesis, even at a concentration of 100 μ*M*, in contrast with other metals examined *(250)*. Interestingly, exposure of adult bonnet monkeys to CrVI added to the drinking water for 6 mo was shown to disrupt spermatogenesis, whereas testicular steroidogenesis was not examined in the study *(251)*. These results differ from those obtained in a study in which several reproductive parameters were compared between Danish welding workers exposed to chromium as well as other heavy metals, and workers not involved in metal industry *(252)*. This study found no statistically significant differences in serum testosterone, FSH and LH levels, or sperm morphology and motility that could be attributed to welding. Thus, the resolution of these apparent contradictions on the toxicity of chromium on testicular steroidogenesis and spermatogenesis will require further studies.

Cobalt

Cobalt is a transition metal, a byproduct of the production of hard metals and alloys, used in the fabrication of hard metal cutting or grinding tools. It is also found in thermal sprays as well as in products used for coloring glass, paints, and ceramics, and in porcelain enamel. Workers in the metal industry are exposed to cobalt, which has been implicated in the development of hard metal lung disease and various respiratory pathologies in these workers. Cobalt is present at low

levels in vitamin B12 dietary supplements. Prolonged dietary exposure of rats for up to 98 d was shown to induce testicular degeneration and thickening of basement membranes, and the authors proposed that it was a secondary response to the testes becoming hypoxic because of blockage of blood supply to the testes *(253)*. By contrast, prolonged dietary cobalt treatment did not induce deleterious effect in sheep testis, as no gross or microscopic testicular lesions were observed and spermatid reserves were unaffected *(254)*. Thus, pathological effects of dietary cobalt exposure appear to be species-related. The effects of exposure to the artificial isotope cobalt-60 (^{60}Co), used as a source of χ-irradiation in cancer radiotherapy, have also been examined in several types of mammals. γ-irradiation with ^{60}Co was shown to induce significant decreases in testis mass and epididymal spermcount in mice *(255)* as well as damage in testicular cells and decreased androgen production in rats and gerbils *(256)*.

Lead

Human exposure to lead is still a public health concern mainly because of the leaded paint in older homes. However, lead also reaches the aquatic environment through industrial and municipal discharges, atmospheric deposition, and weathering processes. All reported effects of lead are adverse to human health. Usually, it inhibits the formation of heme, and thus, adversely affects blood chemistry, affects growth, causes neurological effects because of degenerative lesions, and affects the reproductive system. Lead exposure in men has been linked to abnormalities of spermatogenesis *(257–261)*, decreased free testosterone index (testosterone/sex hormone-binding globulin), and elevated LH, but not FSH levels *(262)*. Moreover, an inverse correlation between circulating lead levels and sperm counts has been reported *(263,264)*. Taken together these results suggest a direct effect of lead to the testis in exposed human.

In vivo exposure of rats to lead suppresses the hypothalamic–pituitary–testicular axis and inhibits various testicular functions leading to reduced testosterone formation and spermatogenesis *(265–268)*. In the rat, lead exposure results in dose-dependent suppression of circulating testosterone levels accompanied by minor or no significant changes in circulating gonadotropin levels *(265,266,269–273)*. LHRH stimulation of lead-exposed rats resulted in increased LH levels, but plasma testosterone levels remained suppressed, suggesting that lead exerts a direct effect on Leydig cell function *(273)*. Indeed, in ex vivo experiments, Leydig cells isolated from rats treated for 5 wk with lead acetate showed a reduced basal and hormone-stimulated testosterone production *(274)*. Direct in vitro treatment of isolated rat Leydig cells with lead acetate also resulted in a time- and dose-dependent inhibition of Leydig cell progesterone and testosterone formation *(274)*. These inhibitory effects of lead on steroid biosynthesis correlated with reduced expression of CYP11A1, 3β-HSD, and CYP17, and altered smooth endoplasmic reticulum *(274)*. More recently, Liu et al. *(275–277)* using as a model the MA-10 Leydig cells confirmed that exposure of the cells to lead acetate inhibits the hormone and cAMP-induced steroid formation and showed that this effect was because of an inhibition of CYP11A1 and 3β-HSD activities. These authors also showed that lead reduced the hormone-induced levels of the 30-kDa mature StAR protein and proposed that lead might also affect cholesterol transport into mitochondria *(275)*. These results are in agreement with earlier morphological and biochemical studies demonstrating that chronic lead exposure results in the accumulation of lipid vacuoles in Leydig cells *(278)* and increase in testicular cholesterol content *(278)*, indicators of reduced cholesterol transport and metabolism. Therefore, caution should be exercised until the levels of the bioactive 37-kDa StAR preprotein are determined. It is possible that lead will accelerate the degradation of the mature StAR protein rather than affect its synthesis. This holds true for all compounds shown to inhibit the hormone-induced levels of the 30-kDa StAR protein, as discussed previously in this review. Liu et al. *(275)* also reported that the calcium channel blocker cadmium abolished the inhibitory effect of lead on steroidogenesis, suggesting that lead might act on a calcium channel tightly linked to the regulation of key steps of the steroidogenic pathway.

Mercury

Mercury is a known toxicant byproduct of human activities. Mercury pollution occurs mostly in water where it is ingested by aquatic organisms, converted to methyl mercury, and concentrated in their tissues. This leads to increasing concentrations in the food chain. Mercury has been found to concentrate in Leydig cells of the testis in exposed human presenting with azoospermia and infertility *(279)*, and in treated rats *(280)*. Methyl mercury exposure alters spermatogenesis and decreases fertility in experimental animals *(281, 282)*. Methyl mercury-induced changes in spermatogenesis did not affect serum testosterone levels in *Macaca fascicularis* monkeys *(282)*. However, chronic administration of methyl mercury resulted in Leydig cell degeneration in rodents *(281,283)*, accompanied by decreased testosterone levels *(283,284)*. Adult rats treated chronically with methyl mercury showed reduced testosterone formation in response to in vivo

Table 1
Chemicals Acting at the LH Receptor Signal Transduction Pathway

Sites of action	*Chemical*	*Use*
LH Receptor-AC-cAMP	Bisphenol A	Plasticizer
	Hexachlorocyclohexanes, lindane	Pesticide
	Ketoconazole	Antifungal
	TCDD	Agricultural and industrial chemical

injections with human choriogonadotropin *(284)*. Decreased steroid formation in response to methyl mercury treatment was shown as a result in part of reduced 3β-HSD activity *(283)*.

METABOLISM OF TESTOSTERONE TO ESTRADIOL

Several environmental agents, including organochlorine pesticides, dioxins, PCBs, and aromatic hydrocarbons can directly disrupt estrogen action leading to developmental and reproductive abnormalities and cancer *(285)*. More recently several metals, including arsenic, cadmium, chromium, and lead were shown to have estrogenic effects and/or disrupt the action of estrogens *(286–289)* and for cadmium, an effect on androgen receptor was shown *(290)*. Considering that Leydig cells can metabolize androgen to estrogen *(291,292)*, and estrogen play regulatory role in male reproductive system and Leydig cell function *(7,293,294)*. It can be hypothesized that some of the effects of the environmental agents presented earlier on Leydig cell steroidogenesis might be mediated by their complementary or antagonistic effects on endogenous estrogen action *(295)*.

Besides the single metal effects on testicular function and Leydig cell steroidogenesis, it should be recognized that we are exposed to mixtures of metals and other environmental chemicals. The impact of such exposure has yet not been assessed. In a recent paper, Veeramachaneni et al. *(296)* reported that early exposure to common chemical contamination in drinking water pollutants results in subnormal mating desire/ability, sperm quality, and Leydig cell function, including hormone-stimulated testosterone formation. More studies will be needed to investigate the impact of multiple exposure through our complex environment on androgen formation by the testis.

CONCLUSIONS

The information presented earlier makes it apparent that the Leydig cell is a common target for numerous pharmacological and environmental agents. The effects of the various chemicals on steroidogenesis can be divided into three categories, including effects on:

1. LH-induced signal transduction pathway.
2. Cholesterol synthesis and transport.
3. Steroidogenic enzymes.

Tables 1–3 summarize the list of the compounds acting at each of those steps. From those tables, it is obvious that the most sensitive steps in steroidogenesis are the cholesterol transport mechanism, and the 3β-HSD and CYP17 enzymes.

The toxicological effects of environmental agents on Leydig cell androgen biosynthesis could have major repercussions for male reproduction and fertility as well as on the function of the androgen-dependent tissues in the body. The impact of such exposure could be even exacerbated by the use of drugs for pharmacological and/or recreational purposes. It has been previously reviewed in detail the pharmacological influence on Leydig cell androgen formation *(10)*. Interestingly most of the drugs that affect Leydig cell steroidogenesis act at the same critical steps in steroidogenesis affected by environmental agents. Thus, agricultural and industrial chemicals should be tested for their effect on Leydig cell steroidogenesis. Recommendations for initial in vitro screening for antisteroidogenic activity of chemicals have been made by the Endocrine Disrupter Screening and Testing Advisory Committee *(297)*. However, this in vitro screening should be complemented by in vivo studies needed to determine the effects of a given chemical or chemicals on the male reproductive system. Measurement of circulating testosterone levels, after 1–3 mo treatment, might not reflect the effects of a compound on testicular function. Measurement of intratesticular testosterone levels before and after hCG-treatment, as an index of the Leydig cell function and responsiveness, should be used when testing new compounds. Moreover, as the end point of testicular function is spermatogenesis, an androgen-dependent process, ideally every new chemical that will be brought

Table 2
Chemicals Acting at Cholesterol Transport to Mitochondria

Sites of action	*Chemical*	*Use*
Cholesterol transport	Econazole	Antifungal
	Miconazole	Antifungal
	Phthalates	Plasticizer
	TCDD	Agricultural and industrial chemical
StAR	Cadmium	Metal
	Dimethoate	Insecticide
	Econazole	Antifungal
	Hexachlorocyclohexanes, lindane	Pesticide
	Lead	Metal
	Miconazole	Antifungal
Mitochondrial integrity	Surfactants	Agricultural and industrial chemical
PBR	PFDA	Plasticizer, surfactant
	Phthalates	Plasticizer

Table 3
Chemicals Acting at Steroidogenic Enzyme Activity and/or Expression

Sites of action	*Chemical*	*Use*
CYP11A1	Dimethoate	Insecticide
	Lead	Metal
	Methoxychlor and metabolite	Insecticide
	PCB	Agricultural and industrial chemical
	Phthalates	Plasticizer
	TCDD	Agricultural and industrial chemical
3β-HSD	Arsenic	Metal
	Cadmium	Metal
	Chromium	Metal
	Hexachlorocyclohexanes, lindane	Pesticide
	Ketoconazole	Antifungal
	Lead	Metal
	Mercury	Metal
	PCB	Agricultural and industrial chemical
	Phthalates	Plasticizer
	Tributyltin, triphenyltin	Biocide
CYP17	Bisphenol A	Plasticizer
	Interferon	Antiviral
	Ketoconazole	Antifungal
	PCB	Agricultural and industrial chemical
	Phthalates	Plasticizer
	TCDD	Agricultural and industrial chemical
	Tributyltin, triphenyltin	Biocide
17β-HSD	Arsenic	Metal
	Cadmium	Metal
	Dicofol	Miticide
	Hexachlorocyclohexanes, lindane	Pesticide
	Ketoconazole	Antifungal
	PCB	Agricultural and industrial chemical
	Tributyltin, triphenyltin	Biocide
CYP19	Ketoconazole	Antifungal
	Perfluorooctanoate	Plasticizer,surfactant
	Tributyltin, triphenyltin	Biocide

into our environment should be tested for their long-term effects on spermatogenesis. Measurement of Leydig cell steroid production in response to a chemical agent could predict alterations of spermatogenesis because of the impaired steroidogenic ability and subsequent reduced testosterone production by the Leydig cells.

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29 The Leydig Cell as a Target for Male Contraception

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SUMMARY

Control of testicular steroidogenesis and gametogenesis represents the main target of hormonal male contraception. The suppression of testosterone as a Leydig cell product and of follicle-stimulating hormone (FSH) is the main target to prevent spermatogenesis. To this end, both leutinizing hormone (LH) exerting its regulatory action on the Leydig cells and FSH, which acts directly on the seminiferous tubules need to be eliminated. To prevent LH and FSH release, gonadotropin-releasing hormone as the sole releasing factor for both gonadotropins, has to be shut down. The release of gonadotropin-releasing hormone and hence the gonadotropins is under a negative feedback control of testosterone. Downregulation of both LH and FSH leads to spermatogenic arrest, but germ cell maturation is restored as soon as both hormones regain their normal values. Although, intratesticular testosterone is suppressed, peripheral testosterone needs to be replaced in order to maintain normal virility. This is best achieved by testosterone itself, which needs to be combined with a gestagen. At present, hormonal fertility regulation through the Leydig cell provides the most promising method for men who wish to control their fertility.

THE NEED FOR A MALE CONTRACEPTIVE

Male contraceptive strategies, if used worldwide, are obligated to achieve certain goals: to halt a male-driven population growth, to help improve the social and medical conditions in developing countries, and to maintain stable populations and socioeconomic standards in developed countries. Ideally, contraceptives should be fast-acting, cheap, easily accessible, safe, and reversible. Currently, men have only three options for contraception: withdrawal, condom, or vasectomy. Broad acceptance of contraceptive methods is not only determined by their efficacy but also by socioeconomic needs and ethnic preferences *(1,2)*. Hence, the spectrum of contraceptive preparations available is important and a serious deficiency prevails in this regard for men.

Gender equality has become increasingly politically important. In particular, in terms of sexual and reproductive health, the World Health Organization (WHO) established a catalog addressing the needs of females as well as of males. One aim is to provide reliable contraceptives also for males, because those preparations that are clinically well-established are only available for females.

It is beyond any doubt that the spectrum of current contraceptive methods needs improvement and extension, and men are increasingly being requested to participate in fertility regulation in order to share the contraceptive burden more equally between women and men. According to a multinational study, men seem to be willing to share contraceptive responsibility *(1,2)*. Altogether, there is an obvious and immediate demand for new methods for fertility regulation in men.

Apart from the need in humans, research on contraceptives also has an increasing impact on the management of wildlife. In contrast to the usual route of medical research, the human might also serve as a model for animals. Although, reproductive research in humans is advanced, the usual methods to control wild species populations still rely on poison or translocation, which are ethically unacceptable. Thus, the adaptation of contraception methods to wild living animals will develop into an alternative solution to control overabundant animal populations *(3)*.

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THE LEYDIG CELL AS A CONTRACEPTIVE TARGET

The Hypothalamo–Hypophyseal–Testicular Circuit

Male contraception is only applicable to the adult organism and, hence, the description of the endocrine regulation of testicular function will focus on the adult male. Hormonal control of ongoing testicular steroidogenesis and gametogenesis represent the main target of endocrine male contraception. Normal testicular function demands delicate and well-controlled interaction between the hypothalamus, the pituitary, and the male gonad *(4)*.

In the 1920s and 1930s of the last century, it had already been recognized that the pituitary gland is imperative for normal testicular function *(5)*. Surgical ablation of the hypophyseal gland provoked testicular atrophy in rats and nonhuman primates. Subsequent work demonstrated that the administration of androgen preparations ameliorated or reversed the inhibitory effects of hypophysectomy on gametogenesis in these species *(6)*. The pioneering work of Greep *(7)* established the dual concept of hormonal control of testicular functions: luteinizing hormone (LH) exerts its regulatory action indirectly through testosterone produced by the Leydig cells and Follicle-stimulating hormone (FSH) acts directly on the seminiferous tubules. This concept is essentially still valid today with the one exception that FSH can also stimulate (indirectly and/or specifically for certain developmental stages) Leydig cell function in the immature male *(8)*.

The hypothalamo–hypophyseal–testicular loop was closed by the following two observations. *(1)* A hypothalamic factor was identified, which was required by the pituitary to ensure the synthesis and secretion of the gonadotropic hormones. This factor, gonadotropin-releasing hormone (GnRH), is a decapeptide *(9), which* is synthesized in the arcuate nucleus of the hypothalamus. Distinct releasing hormones were originally proposed for LH and FSH. However, at present, the available evidence argues in favor of the existence of GnRH as the sole releasing factor for both gonadotropic hormones, although in evolutionary terms 16 different GnRH transcripts have already been demonstrated *(10)*. This view is derived from findings that blockade of GnRH synthesis, immunisation against GnRH, or the prevention of binding of GnRH to its pituitary receptors abolishes the secretion of both gonadotropic hormones (reviewed in refs. *2,4*). The release of GnRH and the gonadotropins is under the negative feedback control of testosterone. In fact, it has been known for long time that the administration of testosterone inhibits testicular function *(11–13)*.

Regarding the regulation of pituitary gonadotropin release and gonadal functions, perhaps the most important feature of hypothalamic GnRH release is the ultradian cycle of approx 50 min (for review *see* ref. *14*), as this secretion pattern is of pivotal importance for the orderly (pulsatile) secretion of the gonadotropic hormones from the hypophyseal gland. Both the frequency and amplitude of secreted GnRH determine the release of LH and FSH, and consequently, a normal pattern of GnRH release is the prerequisite for regular reproductive function *(15)*.

LH and Spermatogenesis

LH is a dimeric glycoprotein containing a glycosylated polypeptide α-subunit and β-subunit that are covalently linked *(16)*. LH binds to specific receptors in the testis which are located on Leydig cells and has a molecular weight of 93 kDa *(17)*. The LH receptor (LHR), like the FSH and GnRH receptor, belongs to the family of G-coupled receptors *(18)*. Common to these receptors are seven transmembrane domains that are crucial for the normal functioning of the gonadotropin receptors. The main action of LH is to bind to its receptor in the Leydig cells and to induce the synthesis and release of testosterone. Testosterone then acts to promote the spermatogenic process in the seminiferous tubules. Production of testicular testosterone depends largely on LH, and testosterone is a *(19)* key regulator of male gametogenesis (*see* page 471, second column).

This concept received compelling support from recent clinical and molecular observations. Single mismatch mutations in the transmembrane region were associated with a constitutive activation of the LHR in the absence of its ligand and patients bearing this LHR defect showed familial precocious puberty, i.e., the premature maturation of the gonad *(20,21)*. As one would expect, this condition could not be ameliorated by LH. A mutation in the sixth transmembrane domain of the LHR is a hot spot for receptor inactivation, but other positions have also been described *(22,23)*. This mutation does not affect human chorionic gonadotropin (hCG) binding, but abolishes ligand-stimulated cAMP production with the clinical consequence of Leydig cell hypoplasia and male pseudohermaphroditism *(24)*. There seems to be no functional necessity for LH in the male until the onset of puberty. The first wave of spermatogenesis in fetal mice occurs without LH or relies on maternal hCG in male fetuses *(22)*. LHR knockout (LHRKO) mice have normal testis at birth and the number of Leydig cells is identical to that of control

animals *(25–27)*. In the search for Leydig stem cells, methods rely on the fact that these are negative for the LHR and therefore do not require LH for the first maturation step *(28)*. After onset of puberty, spermatogenesis is not completely abolished but reduced. This can be rescued by testosterone administration. In the LHRKO mouse many maturing germ cells undergo apoptosis after reaching the early spermatocyte state, so that only some can complete meiosis and undergo spermiation in which they are arrested as round spermatids *(25,26)*. Patients with LHR inactivating mutations and LHRKO mice demonstrate that Leydig cells require LH stimulation to mature *(22,26)*.

It would follow from these observations that suppression of spermatogenesis for the purpose of endocrine male contraception requires inactivating the Leydig cells. Both irreversible consequences and ethical problems prevent knocking out the receptor genetically but elimination or prevention of LH activity remains implicit in this strategy. However, it should be stressed here that any discussion about the role of LH for germ cell development actually means testosterone, as testosterone production is the result of LH action within the testis *(25)*.

Recent work has indicated that LHRs are not confined only, to the Leydig cells within the male gonad. The presence of LH/hCG receptors has been demonstrated in endothelial cells of the testicular microvasculature *(29,30)*. The relevance of vascular LH/hCG receptors for testicular physiology is currently unknown (for review *see* ref. *31*). LH might not be the only factor stimulating testosterone production because in studies administering exogenous LH, pulsatile testosterone production seems to increase before LH reaches the Leydig cells through the interstitial space *(32–34)*. This could be mediated by the vascular endothelium expressing LHRs. Previous studies in the rat reported focal spermatogenic damage, and inflammation-like changes following administration of high doses of hCG (100 IU) or GnRH agonists *(35–37)*. These effects were related to initially (2–6 h after injection) decreased blood flow caused by hCG-induced precapillary constrictions *(38)*. It has also been found that LH first decreases and later increases testicular blood flow and causes polymorphonuclear leucocyte accumulation and macrophage recruitment *(35,39)*. These studies raise the possibility that besides testosterone *(40)* LH might also be involved in the control of testicular blood flow, at least in the rat *(41)*. In the primate, evidence is lacking to suggest that hCG causes testicular damage but in rats hCG administration causes Leydig cells to stimulate inflammation, probably by secreting angiogenic factors *(42)*. The administration of 250 IU hCG on alternate days throughout a 4 wk period to juvenile cynomolgus monkeys had no discernible detrimental effect on testicular morphology *(43)*. Thus, although blood flow was not measured in that study, our findings could not confirm the suspected role of hCG in provoking testicular damage in this primate species *(44)*.

Although, LHRs were believed to be expressed in the gonads only, a number of reports documented the presence of LHRs in a variety of organs and tissues. The rat prostate expresses a functional gonadal-sized receptor capable of binding iodinated hCG and stimulating a cAMP response in a LH dose-dependent manner. The main expression was seen in the ventral lobe, which is highly dependent on androgens. Tao and his coworkers *(45)* have also demonstrated the human prostate to express the LHR. These findings raise the possibility that in the male LH exerts extragonadal actions and influences prostatic functions *(46)* which still remain undefined.

FSH and Spermatogenesis

FSH, like LH, is a glycoprotein with an α-subunit and a β-subunit joined by a covalent bond *(16)*. FSH is the second key factor in the regulation of spermatogenesis and acts directly upon the seminiferous epithelium through a specific receptor that also belongs to the family of G-coupled receptors *(47)*. Unlike the LHR, the FSH receptor (FSHR) is only expressed in the male or female gonad *(48)*. Even within the testis, the presence of FSHRs could be proven unequivocally only in Sertoli cells.

The importance of FSH for the spermatogenic process in the adult, at least in rats, has been questioned in earlier studies because immunisation against FSH did not affect spermatogenesis *(49)*. And FSH in hypophysectomized rats, failed to support germ cell development *(50)* or the survival of germ cells *(51)*. However, studies in adult nonhuman and human primates, hamsters, rats, and bears support the role of FSH for spermatogenesis and suggest its involvement in spermatogenesis *(52–56)*. Many studies in patients with genetic variants of the FSHR have provided models to define the role of FSH in human spermatogenesis and demonstrate the FSH contribution to male gametogenesis *(57–60)*. General acceptance prevails regarding the need for FSH for initiating spermatogenesis in the maturing male *(44,61,62)*.

The establishment of a FSH-β null mouse and a FSHR knockout (FORKO) mouse was crucial for the understanding of FSH function in the mouse testis

(63–65). Adult FORKO male mice exhibit increased levels of FSH and decreased serum testosterone but normal LH values *(65)*. The reduced levels of inhibin and/or testosterone most probably account for the increase in FSH secretion. In FSH-β null mice which retain full FSHR function and normal LH, circulating testosterone remains unchanged as in the wild-type *(64)*, yet these males also experience testicular dysfunction and reduced sperm output. The intercellular communication between Leydig and Sertoli cells in the complete absence of FSHR shows a significant reduction in adult FORKO males *(63)*. FORKO mice have low sperm counts, decreased sperm motility, and aberrant sperm morphology. Flow cytometric analysis of testicular cells exhibits a relative increase in the cells of 2N diploid cells compartment (spermatogonia, Leydig, and Sertoli cells). The proliferation rate of spermatogonia in this knockout mouse remained unaltered but the relative Leydig cell number increased *(66)*. The mere fact that the seminiferous tubule is the target for FSH implies that FSH should be involved in germ cell development. Although, in adult FORKO mice the Sertoli and germ cells are closely associated and the neighbouring spermatocytes or early round spermatids are not altered *(67)*, the seminiferous epithelium shows large dilated spaces surrounding nearby germ cells. The main FSH function in Sertoli cells seem to be helping spermatocytes to mature into round spermatids, but Sertoli cells retain this function in FORKO mice, although spermatid quality control seem to be lacking. FSH supported the formation of round spermatids in hypophysectomized rats *(68)* and the evolution of elongated step 19 spermatids in GnRH antagonist-treated rats *(69,70)*. In FORKO mice the total number of Sertoli cells is reduced and therefore the number of maturing germ cells is reduced in FORKO mice because of the diminished Sertoli cell workload, which is species-specific *(65,71)*.

The importance of FSH for the ongoing spermatogenic process is straightforward in adult primates. Both active and passive immunization against FSH in rhesus monkeys *(Macaca mulatta)*, a seasonally breeding species, specifically neutralized FSH but not LH, the latter evidenced by the unchanged peripheral testosterone concentrations *(70,72)*. Hence, this experimental paradigm proved particularly suitable for investigating the specific role of FSH at least in nonhuman primate spermatogenesis. Following the selective reduction/elimination of FSH, testicular size declined to 50% of baseline and germ cell development was largely interrupted, rendering the animals severely oligozoospermic. Studies in the cynomolgus monkey *(Macaca fascicularis)*, lend support to a role for FSH in the function of the adult testis. Under experimental ablation of gonadotropin secretion, FSH was fully able to maintain testis dimensions and the spermatogenic process *(73,74)*. Further albeit indirect evidence for the ability of FSH to act on adult cynomolgus monkey spermatogenesis was provided by van Alphen and coworkers. They observed that the administration of human FSH to adult and intact cynomolgus monkeys, as well as to rhesus monkeys, doubled the number of germ cells *(75)*. Collectively, these observations indicate a physiological role of FSH during germ cell development in the adult nonhuman primate.

Male FORKO mice can be used to compare testis characteristics with those of nonhuman primates manipulated experimentally and men volunteering for contraceptive studies. The abolishment of FSH or the receptor function leads to poorly condensed DNA packaging in sperm irrespective of the method in humans as well as in various animal models. Although, there is a decrease in sperm counts of more than 70% in FORKO males and in FSH-immunized monkeys, it appears that the effect of the lack of FSH/FSHR signaling on the quality of ejaculated sperm in primates is more dramatic than that observed in rodents *(63,76)*. Thus, it seems that primate sperms are more susceptible to altered Sertoli cell function compared with rodents, but this has not been confirmed by histological analysis of the Sertoli cell workload, which shows no significant differences between rodents and primates including men *(71,77)*.

In addition, an inactivating missense mutation in the extracellular ligand binding domain of the FSHR has been found to be involved in the pathology of hypergonadotropic hypogonadism and amenorrhea in women *(78)*. Detailed analysis of men exhibiting this mutation showed that spermatogenesis can still occur in these patients and proves that men with FSHR mutations can maintain fertility to a certain degree *(60,79)*. Studies of mouse models with null mutations of either the FSH β-subunit (FSHβKO mice) or the FSHR (FSHRKO mice) showed that the Leydig cell number is indirectly regulated by FSH, because FSHRKO mice show reduced Leydig cell numbers. This decrease appears to be induced by the Sertoli cells *(8,80)*.

Administration of hCG to normal men reduces sperm numbers. In contrast, in hypogonadotropic men treatment with hCG alone maintained qualitatively normal spermatogenesis after initial induction with GnRH and hCG/human menopause genadotrophin (hMG) administration *(81,82)*. Thus, although testosterone production by Leydig cells is stimulated under hCG treatment,

and spermatogenesis is completed and qualitatively normal *(82–84)*, testicular involution might occur and sperm counts might decrease, pointing out the need for FSH to maintain the human spermatogenesis quantitatively *(82,85)*. In some patients in whom hCG fails to restore spermatogenesis FSH is reported to normalize spermatogenesis *(86)*. Successful reinitiation of sperm production in hypogonadotropic hypogonadal men requires pulsatile GnRH therapy and hCG, LH, or hCG to achieve normal quality and FSH for normal amounts of the restored spermatogenesis *(82,87)*. It was discovered for the first time in a patient with a constitutionally activated FSHR, who had normal spermatogenesis despite complete hypophysectomy *(88)*. It has become evident *(89,90)* that FSH is a highly trophic hormone for the human testis and, as a consequence, FSH bioactivity or FSHR activity need to be blocked or eliminated if effective endocrine male contraception is to be achieved.

Male Contraception

Relative Importance of LH and FSH for Spermatogenesis

Much has been written concerning the relative or specific functions of LH/testosterone and FSH during various phases of gametogenesis in the mammalian testis *(90–93)*. This discussion involves three major issues.

1. Do LH and FSH exert differential effects during pubertal initiation of spermatogenesis, during maintenance of spermatogenesis and during reinitiation of spermatogenesis once the gametogenic process has been interrupted.
2. Can either hormone alone induce completely normal production of germ cell numbers.
3. Do LH and FSH activate specific genes or induce the production of LH-/FSH-specific testicular factors?

Although, none of these topics has been completely clarified till date, numerous knockout mouse models have provided insight into many features of these questions. It is our current belief that in the intact and healthy male both LH and FSH are physiologically active to ensure sufficient yields of germ cells. Downregulation of both hormones leads to spermatogenic arrest but germ cell maturation is restored as soon as both hormones regain their normal values *(94)*. Quantitatively normal production of germ cell numbers is only guaranteed in the presence of both LH and FSH under normal conditions. It is important to note that in virtually all hormone-replacement studies published so far, the administration schedules and the resulting hormone patterns only mimicked the normal physiological pattern if testosterone had been given as an implant. Oral and transdermal testosterone delivery fail to suppress spermatogenesis in many men.

It is possible that species-specific differences exist in the relative importance of LH and FSH for the spermatogenic process. Sequence analyses of both hormones have demonstrated that the variability is higher than expected from interspecies hyperstimulation experiments. For example, new world monkeys do not express LH because their LHR lacks an exon and the pituitary action depends entirely on choronic gonadotropins (CG) *(95–98)*. The effects of both hormones—LH and CG, which evolved from the LH-β gene and manifest specifically in the anthropoid primates—are mediated by the LHR (*see* ref. *71*). It was assumed that the function of CG in adulthood is more or less restricted to the establishment of pregnancy in female primates and has no function for male reproduction, but examination of the LHR demonstrated the LH/CG dualism in primates to be related to evolutionary processes *(99)*. In the marmoset monkey, LHR exon 10, although present at the genomic level, is not expressed *(100)*. For the human LHR, this exon is necessary for the expression of receptor protein. Interestingly, a clinical case lacking LHR exon 10 has been described *(95)*. This patient had developed a male phenotype but had retarded pubertal development and symptoms of general androgen deficiency that was rescued by hCG administration. Additionally, it was demonstrated that marmoset pituitary expresses CG *(98)*, raising the possibility that marmoset Leydig cells are driven by CG. This lack of LHR exon 10 expression was also found in other new world monkey species confirming that the LHR type II interacting with CG is predominant in New World primates.

However, in old world primates, in hamsters and in bats, spermatogenesis seems to be more dependent on FSH than on LH *(101–104)*, whereas in the rat LH/testosterone are more effective. Overall, LH/testosterone and FSH alone stimulate gametogenesis after meiosis has set in. From the viewpoint of hormonal male contraception, it is clear that both LH/testosterone and FSH in the testis need to be reduced lower than a certain threshold for successful and even reversible interruption of the spermatogenic process.

To examine the role of FSH for the regulation of spermatogenesis, patients with hypogonadotrophic hypogonadism turned out to be a valuable experimental model. These patients are normally treated with pulsatile GnRH (secondary hypogonadism) or with a therapy administering both gonadotropins, where upon spermatogenesis

can be induced and patients become fertile (for review *see* refs. *54,87*). FSH alone or combined with low doses of testosterone was reported not to be sufficient to induce fertility in such patients *(105)*.

However, there are patients with active spermatogenesis and mature sperm exhibiting completely absent LH and testosterone action. It was hypothesized that these men might produce residual low levels of LH below the detection limit but sufficient to induce intratesticular testosterone values that can support spermatogenesis initiation *(106)*. Alternatively, FSH alone might be able to stimulate testosterone secretion in Leydig cells which would provide sufficient steroid amounts *(54,107)*. Nevertheless, to initiate and maintain qualitatively and quantitatively normal spermatogenesis the presence of both gonadotropins—LH and FSH—is simultaneously required *(54)*.

EXTRATESTICULAR ACTIONS OF TESTOSTERONE

It has been already mentioned that the approach to endocrine fertility regulation in men includes the suppression of LH and FSH activity and the simultaneous supplementation of androgens *(94,108,109)*. The latter is absolutely critical to guarantee the well-being of subjects under endocrine fertility control as androgens exhibit a broad spectrum of physiological activities in the male organism *(110–112)*. Developmentally, androgens determine sexual differentiation, male-specific characteristics, body growth, and proportions. However, beyond that, androgens are of pivotal importance for erythropoiesis, muscle and bone anabolism, and libido and sexual appetence. Lipid metabolism is also pronouncedly influenced by androgenic hormones. Androgen deficiency in the adult is associated with osteoporosis, pallor and wrinkling of skin, anemia, muscle atrophy, decreasing ejaculatory volume, and loss of libido *(113–118)*.

Testosterone levels are not solely responsible for the hormone's impact on the entire body or the various organs. The androgen receptor (AR), a steroid receptor which interacts with the DNA to initiate transcription shows polymorphisms (e.g., CAG repeats) modulating its responsiveness. The more triplets that are repeated in exon 1 of the receptor gene, the less sensitive it is for androgen action mediated by the receptor (*see* ref. *118*). The length of the CAG repeats varies among individuals and, thereby, influences transcriptional activity; therefore, the CAG repeat polymorphism of the AR modulates testosterone effects in healthy eugonadal men. Furthermore, the polymorphism has considerable clinical impact because of the pharmacogenetic implications for the treatment of hypogonadal men *(119)*. Thus, serum testosterone levels are only one of many factors forming the cascade of androgen action and all other factors might follow a pattern independent of serum testosterone levels.

In hypogonadal men exhibiting symptoms of lethargy, depressive moods, or sexual inactivity, testosterone therapy significantly improved these features *(120)*. On the other hand, in violent sexual offenders, serum testosterone levels were significantly different *(120)*. It has been proposed that boys' exposure to androgens might not cause violent behaviour but increase violent acts and that this persists up to adulthood *(121)*. Most of the proven knowledge and substantial assumptions in these behavioral aspects derived from studies in animal models, for example exogenous administration of testosterone propionate to nonphysiological levels in cynomolgus monkeys led to a significant increase in aggression, reflected by changes in the social status of the monkeys *(122)*.

In the prospective Baltimore longitudinal study of aging, Harman and colleagues *(123)* reported, independent of health factors, age-related decreases in testosterone and free testosterone leading to a wide range of hypogonadal values in aged men. Furthermore, by analysing total testosterone, free testosterone, and SHBG concentrations in relation to neuropsychological parameters such as verbal and visual memory, mental status, visiospatial ability, and depressive symptoms, the study showed a direct correlation between testosterone levels and cognitive performance *(124)*. The exact assessment of these effects is problematic because of the pivotal role of many other factors such as environment, education, and cultural background. Gender differences in cognitive function tests have been widely reported: men tend to excel in fields of spatial cognition, whereas women show better abilities in verbal fluency *(125,126)*.

THE LEYDIG CELL AND TESTICULAR FUNCTION

It was noted in all species studied so far that testicular androgen concentrations far exceed those present in the circulation *(127–129)*. The testosterone concentration that is required to maintain spermatogenesis quantitatively is far in excess of the concentration required to saturate testicular ARs in intact rats *(130)*. Consequently, the concept was put forward that intact spermatogenesis necessitates high intratesticular levels of androgens. This view had to be revised when

Cunningham and Huckins *(131)* reported that the spermatogenic process was maintained at testicular testosterone concentrations of 5% or even lower than normal, although at a reduced level. These findings were confirmed in many subsequent studies *(132–134)* and triggered research geared to determine how much testosterone is actually needed for normal germ cell development *(92,135)*. In the absence of detectable FSH levels induced by high-dose testosterone or estradiol treatment or immunization against LH or GnRH, 20–40% of normal testicular testosterone concentrations enable spermatogenesis to proceed in a quantitatively normal manner in rats *(136–141)*. However, in the presence of endogenous FSH, the androgen requirement for complete and quantitative germ cell development is considerably lower and quantitatively normal spermatogenesis was achieved with only 10% of testicular androgens compared with control animals *(142)*. The experimental paradigm of GnRH antagonist plus testosterone administration permitted these observations, as androgens selectively stimulate the synthesis and secretion of bioactive FSH *(143)*. Thus, it can be stated that the androgen dependence of gametogenesis is influenced by FSH. Kerr and coworkers *(144)* suggested that the function of FSH is to potentiate the pregametogenic action of testosterone, and Mi et al. *(145)* showed in coculture experiments of chicken germ cells and Sertoli cells that FSH and testosterone together induce stem cell cleavage. Baker and coworkers *(80)* could also show that Sertoli cells determine the Leydig cell number and that constitutive activity within the FSHR is sufficient to stimulate this process in FORKO mice. The testicular androgen requirements for primate spermatogenesis are not precisely known, although the importance of intratesticular testosterone for spermatogenesis has been proven by Coviello et al. *(128)* and previously in an elegant study by Schaison et al. *(105)*. Two groups of five men with hypogonadotropic hypogonadism each were either subjected to administration of testosterone (enanthate) and FSH for 2 yr followed by an LH/FSH containing preparation (human menopausal gonadotropin (+hCG) or vice versa. The LH/FSH preparation induced the appearance of spermatozoa in the ejaculates, whereas testosterone together with FSH were ineffective. Leydig cell function is directly stimulated by hCG raising intratesticular testosterone levels, maintaining spermatogenesis in men with gonadotropin suppression induced by testosterone *(83)*. Additionally, findings in boys with Leydig cell tumors or activating LHR mutations clearly show the importance of testicular androgens for human spermatogenesis *(146–148)*.

Although, the importance of testosterone for spermatogenesis is well-documented, generally it has been difficult to show a direct relationship between testicular androgen levels and testicular germ cell numbers. It has been shown that reduction of intratesticular testosterone is accompanied by an increase of germ cell death and AR redistribution *(149)*. Interestingly, a close correlation between the number of elongated spermatids and seminiferous tubule fluid testosterone was observed in rats *(134,150)*. Hence, the choice of the experimental model seems to be decisive. Is the assessment of testicular testosterone an appropriate parameter for judging the relationship between testosterone and spermatogenesis? DHT is one of the major metabolites of testosterone within the seminiferous tubules *(68,151)*. However, attempts to correlate testicular levels of DHT with the spermatogenic status were unrewarding both in rats *(152)* and in nonhuman primates *(153)*.

MODE OF ACTION OF TESTOSTERONE AND FSH DURING SPERMATOGENESIS

Many studies proved beyond doubt the ability of testosterone and FSH to stimulate germ cell development, but our knowledge of how testosterone and FSH accomplish these effects is still at a hypothetical stage. Receptors for testosterone in the seminiferous tubule are expressed on Sertoli cells and on peritubular cells and it seems logical to assign a decisive role to the Sertoli cell and possibly also to the peritubular cells in mediating androgenic actions during the functional control of gametogenesis. Mice with a Sertoli-cell-specific androgen receptor deficiency (SCARKO mice) are phenotypically almost identical to wild-type mice. However, their testes are atrophied and they are infertile, showing spermatogenic arrest at the diplotene premeiotic stage *(152,154)*. Gene expression studies show that they lack anti-Mullerian hormone, androgen-binding protein, and meiotic genes such as *cyclin A1* and *sperm-1 (56)*. The action of testosterone is a prerequisite for completion of meiosis, which is under strong control of the Sertoli cells.

Although, it has been shown that testosterone in the absence of measurable FSH has the capacity to stimulate complete spermatogenesis *(155)*, testosterone interacts with FSH to achieve quantitatively normal sperm production. In a hypogonadal gonadotropin-deficient transgenic mouse model *(156)*, males stimulated with FSH revealed an increased number of Sertoli cells and spermatogonia independent of testosterone action and established a normal spermatogonia/Sertoli cell ratio. FSH also elevates meiotic spermatocyte numbers, but

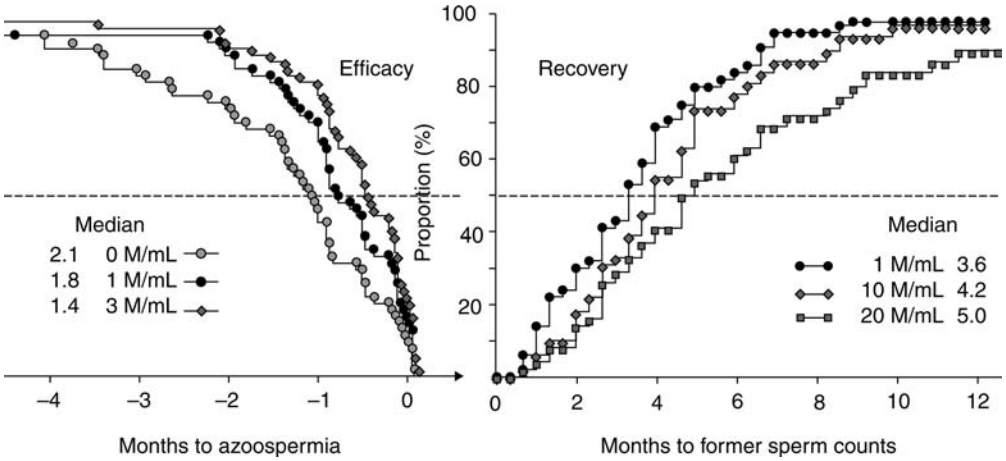


Fig. 1. Formulas of testosterone and gestagens derived from 19-nortestosterone. The synthetic androgen MENT is also shown. (Adaptated from ref. *352*).

induces only limited numbers of postmeiotic haploid cells, which arrest during spermiation. Testosterone does not increase spermatogonial proliferation but highly enhances total spermatid numbers independent of LH *(68,157)*, suggesting the importance of the sex steroid for postmeiotic gene expression. When the effects of either hormone alone or in combination were considered, the combined regimen was always better *(156,158)*. Furthermore, testosterone revealed a striking synergistic enhancement of FSH effect on total spermatid numbers (19-fold higher than FSH alone) *(159)*. In an in vitro study in rats, coculture of Sertoli cells with late pachytene cells showed that testosterone together with FSH leads to a reduced apoptotic cell number *(159)*. This transgenic FSH mouse model has allowed a detailed separation of FSH-only effects in vivo or combined action with testosterone and provided strong evidence that FSH alone affects spermatogenesis by regulation of Sertoli cell and spermatogonial proliferation and, together with testosterone, by stimulation of meiotic and postmeiotic germ cell development

INTERFERENCE WITH LEYDIG CELL FUNCTION IN ORDER TO SUPPRESS SPERMATOGENESIS

As stated at the beginning of this chapter, the endocrine approach to male fertility regulation has seen major advances over the past few years and is closest to realization than all other efforts to safely and reversibly inhibit human spermatogenic function. The major limitations currently imposed on the development and marketing of contraceptive regimens already available stems from liability concerns associated with the administration of hormones to healthy men. Nonetheless, hormonal methods constitute the most advanced and the only realistic approach to male contraception by now.

It has become clear that the inactivation of Leydig cell testosterone production will not be sufficient to suppress spermatogenesis completely in the primate, because FSH in its own right is a very potent stimulator of germ cell development. This was convincingly demonstrated in a nonhuman primate model, the rhesus monkey *(160)*: immunization against testosterone did not cause an impairment of testicular functions. The current approach toward inhibition of testicular function relies on the suppression of both LH and FSH secretion and, in parallel, the supplementation of androgen to avoid the symptoms of androgen deficiency (reviewed by ref. *11*). Ideally, testosterone alone should fulfil both tasks of suppressing gonadotropins and preventing symptoms of androgen deficiency. A critical issue then is the availability of long-acting androgen preparations to make their use feasible and practical and the provision of androgen levels lower than the testicular threshold concentrations that would hormonally stimulate germ cell development.

Exogenous Testosterone Alone

It has been known for a long time that testosterone alone is capable of suppressing testicular function in men *(13,161–163)*. Testosterone and its derivatives (Fig. 1) inhibit the release of FSH and LH, the latter effect being associated with a reduction of intratesticular androgen levels in men *(164)*. Simultaneously, the circulating concentrations of testosterone are maintained, thus, avoiding the clinical symptoms of androgen deficiency. Hence, in principle, from the viewpoint of endocrine male contraception, testosterone is the ideal contraceptive for men. However, testosterone

when taken orally, is degraded rapidly in the liver and very high daily doses of 600 mg or more would be required to elicit biological effects *(110)*. This makes their use impractical and such high doses might not remain without toxic side-effects if taken on a long-term basis. 17α-alkylation of the testosterone molecule diminishes testosterone degradation in the liver. However, these testosterone derivatives, for example, 17α-methyltestosterone and fluoxymesterone, were not very effective in suppressing testicular function and 17α-alkylated androgens bear the risk of liver toxicity *(165)*. Testosterone undecanoate (TU), a 17β-alkylated orally effective testosterone preparation, also failed to interfere consistently with germ cell production, because only one of seven volunteers in a contraceptive clinical trial became azoospermic. *(94)*.

A possible explanation for the low effectiveness of these testosterone preparations in suppressing testicular function relates to their serum pharmacokinetics: a sharp peak after ingestion followed by a rapid decline of serum testosterone concentrations, whereby the vehicle (different oils) does not interfere *(166)*. Serum half-life could be successfully prolonged by esterification of testosterone in position 17 and the absorption half-lives increased with increasing length of the ester chain of these injectable preparations *(167)*. Among the various derivatives that have been synthesized, TU, testosterone enanthate (TE), the anabolic steroid 19-nortestosterone-hexoxyphenylproprionate, and testosterone buciclate have undergone clinical testing for endocrine male contraception. A 70% success rate in terms of complete suppression of sperm production was attained with the injectable 19-nortestosterone preparation *(92,94,168, 169)*. The remaining volunteers exposed to 19-nortestosterone were oligozoospermic and among those men given TE, about two-thirds also became azoospermic. Further studies revealed that 90% or more of Asian men responded with azoospermia to TE alone or in combination with gestagens (reviewed in total by ref. *167*).

However, TE has two major disadvantages precluding its general use as a male contraceptive: the need for frequent injections (every week) and the pharmakokinetic profile with initially elevated and unphysiological testosterone concentrations *(170,171)*. The search for improved testosterone preparations with a smooth pattern and prolonged duration of hormone release has identified several preparations of interest. Nowadays, androgen can be applied as injectable testosterone ester in oily vehicles, implants, transdermal systems, buccal, and oral preparations (reviewed in ref. *94*). Testosterone buciclate was shown to maintain serum testosterone levels in orchidectomized nonhuman primates within the normal range for about 4 mo following a single injection of 40 mg *(172–176)*. Clinically, 600 mg testosterone buciclate were administered to hypogonadal men and were found to provide adequate androgen supplementation to these patients without adverse side-effects *(167)*. Terminal elimination half-life and mean residence time were 29.5 d and 65 d, respectively, compared with 4.5 d and 8.5 d for TE *(177)*. Pharmacokinetics show an optimal injection interval of 2–3 wk at a dose of 200–250 mg, but the serum testosterone level oscillates around the normal range *(178)* whereas, given weekly, constant supraphysiological values between 40 and 80 nmol/L were achieved *(179)*. Testosterone buciclate has been tested in a contraceptive study *(176)*. With a single injection of 600 mg, sperm production was not suppressed; using 1200 mg, spermatogenesis was suppressed to azoospermia in three out of eight volunteers. Thus, testosterone buciclate can reversibly and completely suppress human spermatogenesis, but it remained to be seen whether higher doses would yield a better suppressive effect on gametogenesis. Apart from the examination of the long-term half-lives of the injectable testosterone buciclate, no further studies contributing in terms of male contraception have been performed *(180)*, and this ester has not been developed further.

TU provides a comparable half-life and was therefore examined in several studies either given alone *(166,181–188)* or in combination with different gestagens *(94,181,183,185,189,190)*. An injectable formulation of TU was tested in the nonhuman primate *(166,191)* and in hypogonadal Klinefelter patients *(187)*. In a recent study in monkeys by Wistuba et al. *(166)* TU was also tested in different oily formulations, such as castor oil, tea seed oil, and soy been oil, because certain vehicles have not been approved in different countries. No significant differences were found, so the effects appear to be predominantly because of the hormone ester. At a comparable dose of pure testosterone contained in two ester preparations, TU produced only moderately elevated serum testosterone levels and a fourfold longer residence time and a 2.5-fold longer terminal elimination half-life than TE. In clinical studies on male contraception testosterone undeconate was used in men of different ethnic backgrounds (Chinese and Caucasian) with variable results *(183,186)*. In Chinese men, a dose of 1000 mg every 4 wk achieved 100% azoospermia *(182)*, but half the dose was not sufficient for 2% of 290 subjects *(181)*. The high dose given to Chinese men every 8 wk still resulted in 80% azoospermia *(186)*. However, in Caucasian men the high dose administered every 6 wk only achieved azoospermia in 60% of the men *(192)*. These results

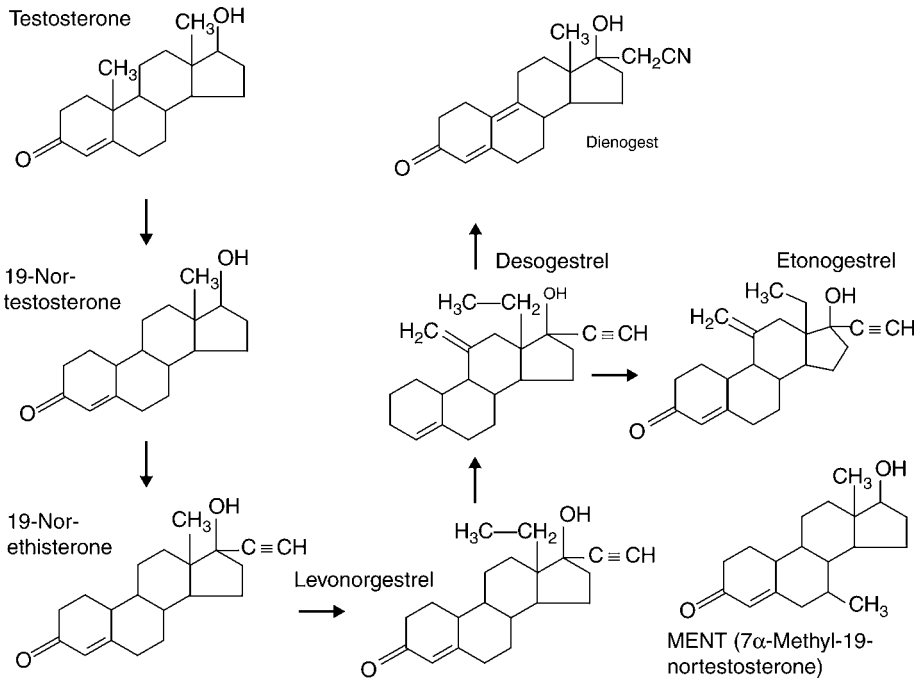


Fig. 2. Results of a male contraceptive efficacy study in 55 men using a combined androgen TE/progestin (DMPA) depot demonstrating high contraceptive efficacy and a complete recovery of spermatogenesis. The left panel shows when volunteers achieved azoospermia (○), 1×10^6/mL (•), and 3×10^6/mL (◊). The right panel plots times to recovery to various sperm concentration thresholds after cessation of the last treatment. Data for the times when men reached sperm densities of 1×10^6/mL (•), 10×10^6/mL (◊), and 20×10^6/mL (■) are plotted.The *dotted line* represents the median time to reach the threshold.

indicate that although ethnic differences might be accompanied by different grades of azoospermia, no complete downregulation of human spermatogenesis can be achieved by testosterone alone given in a physiological range.

Another very promising androgen is the synthetic drug 7α-methyl-19-nortestoterone (MENT). In the primate model it is 10 times more potent than testosterone alone to suppress gonadotropin release *(193)*. The anabolic effects of MENT on the skeletal muscle are also more prominent, which makes it a good candidate for hormone replacement therapy *(194)*. Also in its favor, MENT is not 5α-reduced, so that unlike testosterone it has minimal effects on the prostate *(192,195)*. In male bonnet monkeys, all dosages used (25, 50, 100, 300, and 1000 µg/d) led to complete azoospermia and all monkeys were infertile *(196)*. In clinical male contraceptive studies performed so far, acetylated MENT was given in implants to volunteers in three different doses *(197)*. In the eight of 12 volunteers given the highest dosage, i.e., four implants per subject, azoospermia was achieved but this group also had two nonresponders. Dose-related increases in serum MENT levels and decreases in testosterone, LH, and FSH levels were observed. Generally, side-effects seen with androgen administration, such as increases in erythrocyte count, hematocrit, and hemoglobin, and a decrease in SHBG, were reversible. Taken together, the results in studies using MENT to suppress gonadotropin release are promising, but more clinical trials are needed to demonstrate its efficacy.

Gestagens in Combination With Steroids

Gestagens are efficient and potent inhibitors of gonadotropin secretion and have been tested in contraceptive trials *(168,198–200)*. Obviously, gestagens alone are not suited for that purpose because severe androgen deficiency would result from their administration. More than 40 clinical studies was undertaken to identify an effective combination of the best gestagen and the best androgen (reviewed in ref. *201*, *see* also Figs. 2 and 3). Early studies were directed to TE or cypionate in combination with depot-medroxyprogesterone acetate (DMPA). However, the achieved rates of azoospermia of about 50% were not striking, aside from an effective study in Indonesian men *(202)*. The combination of oral gestagens along with transdermal testosterone preparations proved highly effective and azoospermia was seen in all 12 patients enrolled in the study *(168)*. Originally, the combination of DMPA with 19-nortestosterone appeared particularly effective, induced complete

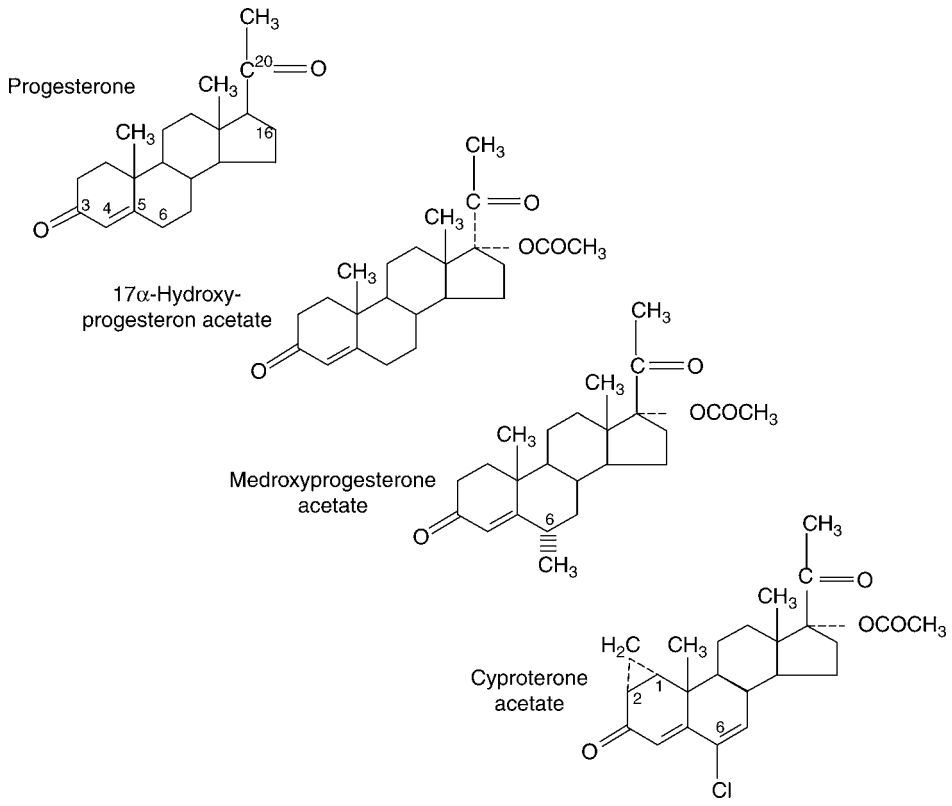


Fig. 3. Gestagens derived from testosterone used in male contraceptive trials.

suppression of sperm production in nine out of 12 men and rendered them severely oligozoospermic *(203)*. Promising results gave impetus to many further studies with testosterone implants or newer formulations such as testosterone undecanonate but in all men only nine reported azoospermia ($<0.1 \times 10^6$ sperm/mL) *(201,204–210)*. Actually, most of the trials in which azoospermia was achieved were performed with Caucasian volunteers, demonstrating that differences in testicular organization *(94)* might explain why Asians might respond better to testosterone alone. Among the numerous different gestagens (for review *see* ref. *211*) used for male contraceptive studies, the most effective at the moment seem to be desogestrel, and norethisterone enanthate. The first was used in combination with injectable TE or implantable testosterone pellets.

Estrogens are highly efficient inhibitors of the hypothalamic-hypophyseal-testicular axis *(212–214)*. Aside from their negative feedback action at the level of the hypothalamus and pituitary, direct inhibitory effects on the testis are likely *(215,216)*. Clinical testing of estrogenic hormones in contraceptive trials was performed using mestranol (estradiol-3-methylether) and estrone. Administration of estradiol preparations suppressed sperm production *(217)*. Briggs and Briggs *(218)* combined mestranol and methyltestosterone, and observed complete cessation of spermatogenesis. In contrast, estrone was ineffective and failed to induce a clear-cut and consistent interruption of gametogenesis *(219)*. Treatment of a trans-sexual male with ethinyl estradiol reduced sperm motility and sperm numbers *(220)*. In rats a clear dose-related effect of estradiol 17-β was demonstrated *(221)*: at a low dosage (0.1 µg/kg/d) estradiol significantly reduces sperm motility, but does not seem to affect serum LH, FSH, PRL, or testosterone levels, testis weight, and size of accessory sex organs. A 100-fold increased dose also reduces serum LH, FSH, weights of testes, and accessory sex organs. The histology of the testes showed disorganization of the seminiferous tubules, vacuolization and absence of lumen, and compartmentalization of spermatogenesis. Tamoxifen, a mixed estrogen antagonist and agonist, was suggested as a male contraceptive in rats *(222,223)* as a result of the strong effects of estradiol on male fertility. Oral application of tamoxifen given in honey to bonnet monkeys did not alter serum testosterone levels or sperm motility but increased the sperm count, contrary to expectation *(224)*. This possible sperm production enhancement has led to an approach for male infertility to increase pregnancy rates by tamoxifen *(225,226)*.

Estrogens persisting in the environment, for example, phytoestrogenic substances in food such as isoflavones from soy-rich diets or estrogenic chemicals, for example, bisphenol A used as softeners delivered by industry and accumulating through the food chain were suspected to cause male infertility and decreasing sperm counts *(227)*. Up to now, no study could prove this estrogen hypothesis in humans. In male rats administration of exogenous estrogenic compounds induced malformation of the reproductive tract and has adverse effects on spermatogenesis, but this phenotype was rescued by increasing androgen levels. In conclusion, these findings indicate that the balanced state between estrogen and testosterone is crucial for reproductive development and fertility rather than the endocrinological effects of the hormones as such *(228,229)*. Against this background estrogens can also be considered as "male hormones" *(230)* and in rats, intrauterine administration of the estrogenic compound bisphenol A even led to slightly stimulatory effects on Sertoli cell number and testis weight *(95)*. The administration of estrogens, might disturb the physiologic equilibrium between estrogen and androgen if the ratio of free testosterone to estradiol reaches a critical level, as in some aged men *(231)*; the patients might show signs of hypogonadism *(232)*.

In general, the administration of estrogenic compounds is associated with serious side-effects such as reduced libido, alteration of blood-clotting factors, and gynecomastia. According to Neumann et al. *(233)*, the symptoms of gynecomastia could not be alleviated despite concomitant substitution with an androgen. In summary, the clinical use of estrogens for suppression of male gonadal function is obviated by the estrogen-related untoward side-effects.

An evaluation of the combined effects of testosterone and estradiol released from silastic implants in the rhesus monkey *(234,235)* yielded an important finding: although sperm numbers in the ejaculate were not reduced to zero, albeit being very low, the animals were rendered infertile in mating tests with receptive females. This study gave the first indication that azoospermia is not necessarily a prerequisite for the endocrine induction of infertility in a primate (this issue is discussed later on page 427). In a clinical trial Handelsmann et al. *(236)* treated 12 male subjects with combined testosterone and estradiol implants to determine efficacy. Besides the side-effects of estrogens discussed earlier, an extra benefit in reducing fertility in men was found. However, while the lower estradiol dosage had minor suppressive effects on sperm parameters, the higher dose produced adverse effects of androgen deficiency. Therefore, the authors do not recommend using this approach as a male contraceptive.

Antiandrogens in Combination With Androgens

The use of antiandrogens has also been considered as a potential approach to control male fertility. The underlying rationale is that testicular and epididymal functions are androgen-dependent and that a blockade of the AR in these organs by an antiandrogen might interfere with the functions of these organs. Nonsteroidal antiandrogens such as cyproterone or flutamide are pure antiandrogens, i.e., they merely occupy the AR in the target organs but are devoid of gestagenic activities *(237)*. Experimental and clinical studies demonstrated that flutamide is unable to suppress testicular functions *(237–240)*. The occupation of the ARs in the brain centers governing hypothalamic and pituitary function through the testosterone feedback circuit are interrupted, leading to a hypersecretion of LH and testicular and serum testosterone *(238,241,242)*. It is believed that the high intratesticular androgen levels displace flutamide from the AR *(243)*. Peripherally selective antiandrogens such as casodex *(237)* were claimed to block only the peripheral, but not the central ARs, thus avoiding the hypersecretion of LH and testosterone associated with the use of flutamide. However, experiments in the rat could not confirm this claim and testicular function remained basically unaffected in two separate studies *(238)*. Another nonsteroidal antiandrogen, anandron, was ineffective in the rat, but the addition of 1 μg estradiol neutralized the central stimulatory effect and potentiated the antiandrogen action *(244)*.

Cyproterone acetate is a steroidal antiandrogen and, in addition to its antiandrogenic properties, has pronounced gestagenic activity *(200,245)*. Hence, cyproterone acetate not only blocks the AR but directly inhibits gonadotropin release *(236,239,246)*. Cyproterone acetate suppressed testicular function in sexual delinquents, but at doses that were associated with a loss of libido and potency and thus unacceptable for male fertility regulation. Reduced doses of 5–20 mg/d were tested in clinical trials for male contraception and sperm counts and sperm motility were reduced *(247–249)*. Serum testosterone levels were reduced lower than normal and side-effects such as fatigue and decreased libido and potency were reported. A combination of steroidal antiandrogens with androgens for male contraception was proposed, i.e., the gestagenic activity of the antiandrogen would suppress pituitary gonadotropins and thus testicular function, and the supplemented androgen would prevent androgen

deficiency. The feasibility of this approach has been tested clinically *(207,208,249–251)*. The addition of cyproterone acetate (25 or 100 mg/d) to a TE administration regimen (100 mg/wk) induced azoospermia in 10/10 men within 6–8 wk, whereas, under TE alone 3/5 men developed azoospermia *(252)*.

DOES ENDOCRINE MALE FERTILITY REGULATION PROVIDE CONTRACEPTIVE SAFETY?

An important question became evident from the studies on endocrine male fertility regulation, i.e., what is the contraceptive efficacy of these treatment regimens? This issue is crucial because male contraception should ensure temporary infertility. A multicenter study with 10 centers in seven countries was launched by the WHO which enrolled 271 healthy and fertile men. Each subject received weekly administration of 200 mg TE *(253)*. Within 6 mo, the suppression phase, 157 out of 225 subjects still participating consistently produced azoospermic ejaculates. These couples entered the efficacy phase and during the following 12 mo did not use any other form of contraception. Altogether, in the 1485 mo of this study one pregnancy was encountered. This investigation demonstrated for the first time, the contraceptive efficacy of an endocrine male contraceptive regimen. The safety index of 0.8 is high, compares favorably with female injectable contraceptives (0.2–0.4), and is superior to the female pill *(3)*. Overall, 30% of volunteers in the earlier-mentioned study did not qualify for the efficacy phase as their sperm counts did not drop to zero. However, these men produced oligozoospermic ejaculates, raising the issue of whether oligozoospermia provides contraceptive efficacy.

In order to address the question more systematically, WHO initiated a second multicenter study in a total of 16 centers in 10 countries involving 670 volunteers, of whom 205 were East-Asian and 465 of non-Asian origin, receiving weekly intramuscular injections of 200 mg TE *(252)*. Of these men, 646 suppressed to severe oligozoospermia, defined as sperm counts less than 3×10^6/mL ejaculate, and entered the 12-mo efficacy phase during which TE was the sole means for fertility control. Among these men, 77 developed consistent azoospermia, yielding a pregnancy rate of 0 per 100 yr (95% confidence interval: 0–1.6). This study and the aforementioned investigation *(248)* proved an important question, which is, that the laboratory diagnosis of azoospermia equates with contraceptive safety. Severe oligozoospermia was associated with an overall contraceptive safety factor of 1.4 (95% confidence interval: 0.4–3.7) *(199)*. In conclusion, a contraceptive method based on regular testosterone injections can suppress spermatogenesis to azoospermia or severe oligozoospermia in 97% of men, regardless of their ethnic origin. However, all attempts addressing Caucasian men failed to identify the parameters precisely predicting which men (one-third do not respond totally) fail to respond to the testosterone-only formulation, but also require a progestin to reach azoospermia *(254)*. The responder–nonresponder question is currently under debate. Severe oligozoospermia can provide a degree of contraceptive protection far better than that of the condom and in fact, comparable with the failure rates of reversible female methods. In 399 normal, healthy, fertile men receiving a weekly injection of 200 mg TE, four pregnancies occurred during 49.5 person-years involving men with oligozoospermia ($0.1–3 \times 10^6$/mL) and none during 230.4 person-years in azoospermic men *(253)*. Analysis revealed that the pregnancy rates (calculated per 100 person years) were closely related to sperm number in the ejaculate, thus, reinforcing the proposition that sperm production should be completely or largely suppressed for the purpose of safe and effective male contraception. These data also clearly demonstrate the feasibility of endocrine male fertility regulation. A major setback of hormonal male contraception is the time gap between start of the therapy and efficacy of these hormonal contraception methods. As in vasectomy, a man wanting to prevent pregnancies with his partner has to wait for over 1 mo before they can solely rely on it. Other side-effects such as lower high density lipoprotein (HDL) and long-term impacts on the cardiovascular system might occur, which need to be addressed in appropriate studies.

NONUNIFORM SPERMATOGENIC RESPONSE TO ENDOCRINE MALE CONTRACEPTION

Participants of the WHO multicenter studies were from Caucasian and Asian populations *(254,255)*. Quite interestingly, the split analysis revealed that more than 95% of Asian men responded with azoospermia, compared with about 65% of Caucasian men, an effect that became evident with the duration of TE administration (reviewed by ref. *256*). These clear differences in the contraceptive response to steroids might be, because of the differences in a decreased spermatogenic potential *(210,257)*, to an earlier and more marked suppression of LH secretion by exogenous androgens in Asian men *(258)*, to different environmental factors

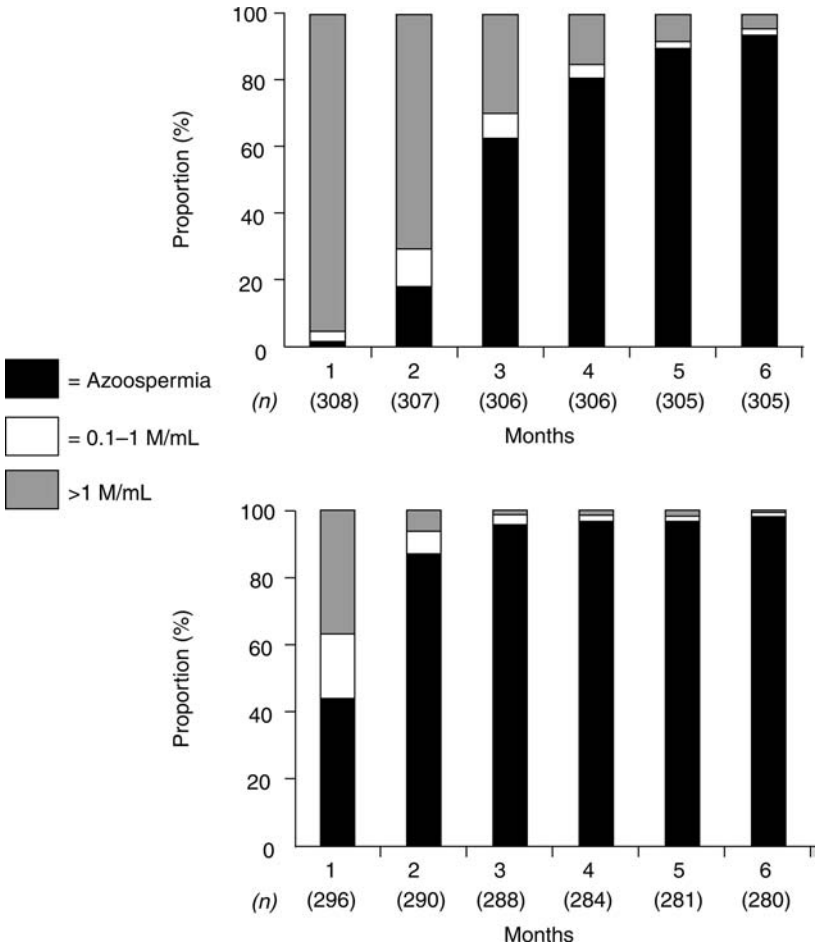


Fig. 4. In a WHO-supported contraceptive phase II multicenter trial, Gu and coworkers *(182)* used monthly injections of TU alone in 308 healthy Chinese men. The upper panel shows the proportion of categorized mean sperm concentration ($\times 10^6$/mL) during the first 6 mo, the lower panel shows the proportion of categorized mean sperm concentration ($\times 10^6$/mL) during the efficacy phase. Numbers in *parentheses* indicate the absolute number of subjects at each time point. ■, 0; □, 0.1–1.0; ▩, 1.1–3; ▥, >3. The results proved that after an initial loading dose of 1000 mg monthly, TU injection at a dose of 500 mg to effectively, safely, and reversibly suppress sperm production in Asian men. Volunteers with sperm counts $\leq 1 \times 10^6$/mL did not induce pregnancies.

such as different diet *(199),* and to different genetic factors such as the CAG repeat numbers of the AR *(259)*. Furthermore, differences in physical and sexual activity among Asian and Caucasian men might also influence reproductive endocrinology.

However, it is important to note that a nonuniform testicular response (responders and nonresponders in terms of the achievement of azoospermia) to testosterone formulations is common in both ethnic groups *(257)*. The nonuniform suppression of sperm numbers in the first WHO study *(252)* could not be attributed to anthropomorphic, ethnical, or androgenic differences nor to compliance *(248)* (Fig. 4). In that study and in a subset of Caucasian men from that study *(261),* it was noted that the rebound secretion of gonadotropic hormones during the recovery period was more pronounced in the responders compared with the nonresponders. This could possibly indicate subtle differences in the endocrine suppression of pituitary and testicular functions.

ANALOGS OF GnRH

The amino acid sequence of GnRH was discovered more than 30 yr ago *(9,262)*. Most important are the amino acids in positions 1–3, which account for the hormone-releasing activity and those in positions 6 and 10, which determine binding to the GnRH receptor (reviewed by ref. *262*). All higher vertebrates, including man, express at least these two GnRH receptor isoforms (I and II). GnRH II differs from GnRH I by three amino acids and is distributed in the body tissues

(263–266). However, the human gene homolog of this receptor has a frame-shift and stop codon, and it appears that GnRH II signaling occurs through the type I GnRH receptor *(262)*.

Agonists and antagonists of GnRH bind to the same receptor but exert a differential effect on GnRH receptor numbers and gonadotropin release. GnRH agonists first upregulate but subsequently downregulate the GnRH receptor, leading to refractoriness of the gonadotrope to the stimulatory effects of GnRH and GnRH agonist (reviewed by ref. *262*). In contrast, the GnRH antagonists occupy binding sites that differ from, but overlap the agonist binding pocket and merely cause the cessation of gonadotropin release by competitive occupancy of the receptor (reviewed by ref. *267*). Pretreatment of pituitary membranes with proteolytic enzymes decreased binding of labeled antagonist more than that of labeled agonist. This indicates the agonist binding site to be less accessible and more buried within the receptor molecule than the antagonist binding site. One piece of evidence for this mechanism derives from autoradiographic studies using radiolabeled GnRH analogs. Agonists of GnRH *(268)* were internalized within minutes, whereas antagonists of GnRH were still present on the gonadotrop membrane several hours later *(269–271)*. GnRH blockade by agonists has been demonstrated in many animals *(268, 273–278)*. The exact mechanism of suppression is still unclear. The agonist decapeptyl downregulates LH in male but not in female rats, suggesting an incomplete agonist downregulation *(279)*. GnRH antagonists completely inhibit GnRH-induced GnRH receptor gene expression, leading to pituitary suppression *(280)*. The competitive nature of GnRH antagonist-induced blockade of primate LH secretion is furthermore suggested by the observation that exogenous, synthetic GnRH was able to displace the GnRH antagonist in a GnRH dose-dependent manner under in vivo conditions *(77,262,267,281–286)*. Substances acting antagonistic to GnRH in the human can be GnRH agonists in other species, for example, in the chicken (reviewed by ref. *287*). Unlike the GnRH agonists, antagonists of GnRH are capable of immediately suppressing gonadotropin synthesis and release within several hours of administration, whereas under GnRH agonist treatment a period of 1–2 wk is required until inhibitory effects on the endocrine system become apparent (reviewed by ref. *288*).

As a consequence of GnRH analog-induced gonadotropin deficiency, the synthesis and secretion of gonadal steroids (in both sexes) is compromised and suppression of testicular and ovarian function is induced *(199,287)*. Thus, they are candidates for a hormonal male contraceptive.

Testosterone in Combination With GnRH Agonists

GnRH agonists initially stimulate LH and FSH secretion for about 2–3 wk. Subsequently, gonadotropin release is inhibited, particularly that of LH *(289)*, and consequently serum testosterone concentrations fall into the range found after orchidectomy. Unfortunately, the outcome of the clinical trials was disappointing *(290–301)*. Only in about 30% of volunteers could spermatogenesis be suppressed to zero or lower than 5×10^6 sperm/mL ejaculate. In one-third of men, sperm counts fell less than 30×10^6 sperm/mL ejaculate, whereas in the remaining participants sperm production was only marginally affected or remained unaltered.

Because in most of the aforementioned studies in which androgen supplementation was provided, the low efficacy of the GnRH agonists was related to the stimulatory effect of testosterone on spermatogenesis. This explanation was supported by the observation that concomitant testosterone substitution counteracted the GnRH agonist-induced spermatogenic involution *(302)*, whereas the provision of testosterone at a later phase (once some degree of spermatogenic reduction had been achieved), did not interfere with GnRH agonist action *(277,289)*. Moreover, subsequent studies identified another likely reason for the low efficiency of GnRH agonists as inhibitors of testicular function. With GnRH agonists the suppression of immunoactive FSH secretion was incomplete or only transient *(303–305)* and, more importantly, FSH bioactivity in serum remained nearly unaltered *(306,307)*. Similarly, in patients suffering from pituitary tumors, GnRH agonist therapy rendered LH/FSH and α-subunit levels unchanged *(308)* or even had a stimulatory effect *(289,309)*. Behre et al. *(310)* evaluated a combination of 19-nortestosterone and GnRH agonist depot. Testosterone treatment preceded the onset of GnRH agonist treatment in order to provide initial suppression of the pituitary–testicular axis. However, even under these conditions, the GnRH agonist stimulated FSH secretion in a GnRH agonist-dose dependent manner and, hence, counteracted the inhibitory effects of 19-nortestosterone. Therefore, GnRH agonists are not suitable as male fertility regulating compounds. These observations reinforce the principle, that effective suppression of primate spermatogenesis requires inhibition of both LH secretion (Leydig cell function) and FSH secretion.

Testosterone in Combination With GnRH Antagonists

Aside from their rapid antigonadotropic activity, GnRH antagonists markedly reduce both immunoactive *(311–314)* and bioactive FSH secretion *(267,315–320)* with FSH bioactivity being even more affected than immunoactivity *(321)*. Clearly, GnRH antagonists are superior to GnRH agonists in term of interfering with the release of both gonadotropic hormones. Unlike the GnRH agonists, GnRH antagonists completely and reversibly inhibit testicular function without any deleterious side-effects *(69,268,142,321–326)*. The GnRH antagonist-induced germ cell loss has been shown to involve the activation of programmed cell death *(326,327)*. Leydig cells, peritubular cells, and Sertoli cells were not apoptotic *(325)* but Tapanainen et al. *(328)* found in rats suppressed by GnRH antagonists that interstial cells did show DNA fragmentation. In contrast, in the macaque the premeiotic germ cells are mainly affected through inhibition of mitosis and not through prevention of meiotic steps *(325)*.

The experimental paradigm of GnRH antagonist combined with testosterone revealed a substantial difference in the feedback regulation of FSH synthesis and secretion by androgens between rodents and primates. In nonhuman primates *(329)* and in men *(330)* testosterone substitution did not interfere with the GnRH antagonist-induced suppression of FSH secretion. In sharp contrast, testosterone maintained *(142,331)* or restimulated *(332)* pituitary FSH synthesis and its release in the GnRH antagonist-treated male rat. At the molecular level, testosterone selectively induces the production of the FSH β-subunit expression *(142,333,335)*. The FSH molecule produced under this condition is bioactive *(320)* and this effect is induced by androgens (testosterone or dihydrotestosterone) but not by estradiol *(336)*.

GnRH antagonists induce effective and reversible suppression of testicular steroidogenesis and spermatogenesis in the nonhuman primate *(325,337)*, a finding that has been confirmed in all subsequent preclinical investigations *(56,74,153,338–341)*.

GnRH agonists are generally characterized by a single amino acid modification in position 6, thereby, enhancing receptor binding and resistance against enzymatic degradation. In contrast, GnRH antagonists have 4–7 modifications *(342,343)*. The clinical development of GnRH antagonists proved extremely difficult, because the available GnRH antagonists with sufficient antigonadotropic activity elicited undesirable side-effects, in particular local skin irritations at injection sites *(339)*.

Cetrorelix is as potent as Nal-Glu but, unlike Nal-Glu, exhibited a favorable benefit-to-risk ratio in clinical trials. Preclinical studies in the nonhuman primate model revealed that cetrorelix very effectively and reversibly suppresses pituitary and testicular functions *(344,345)*. The GnRH antagonists developed more recently such as acyline or abarelix used in trials for prostate cancer patients showed even more promising results *(344)*. In a trial with 170 men receiving an injection of 100 mg aberelix, castrate levels were demonstrated and testosterone was less than 5 nmol/L after 1 d *(345)*. Application of 75 mg/kg bodyweight acyline maintains suppression of FSH and LH for 48 h. Acyline seems to be the most potent GnRH antagonist with a longer half-life demonstrated in a comparison of antide, Nal-Glu, cetrorelix, antarelix, and acyline *(346)*. The action of this antagonist has a nadir of approximately three times the length of similar doses of the other antagonists *(199)*. The biggest drawback of GnRH antagonists in terms of male contraception is its short half-life (*347*, *see* also ref. *339*).

Currently, apart from the long-term acting degarelix, GnRH antagonists need to be administered on a short-term basis to provide a degree of pituitary and testis suppression compatible with azoospermia. A contraceptive regimen requiring daily injections is obviously unacceptable and no reports of degarelix in humans have been reported. In the nonhuman primate, cetrorelix was given for a period of 7 wk and a high dose of testosterone buciclate was injected during week 6 *(348)*. The spermatogenic status present at the inception of testosterone injections (assessed from the number of ejaculated sperm and flow cytometric determination of cell numbers in a testicular biopsy) was maintained by testosterone alone but azoospermia was not reached. The clinical effort was also unsuccessful because cetrorelix-induced azoospermia could not be maintained with 19-nortestosterone alone, and sperm reappeared under continued 19-nortestosterone treatment following withdrawal of the GnRH antagonist cetrorelix *(349)*. However, it has been observed that the inhibitory effects of 10 mg cetrorelix/d given for 5 d on gonadotropin and testosterone secretion could be maintained by a daily dose of 1 mg cetrorelix only *(350)*. In another clinical trial, 15 volunteers with normal semen parameters were treated with 100 mg TE weekly, combined with a daily dose of 10 mg of the GnRH antagonist Nal-Glu subcutaneously for 12 wk to induce azoospermia or severe oligozoospermia. After 12–16 wk, 10 men were azoospermic and another three subjects had sperm counts of less than 3×10^6/mL. The 14 men who were suppressed on combined treatment were maintained on

TE alone for an additional 20 wk. Thirteen of 14 subjects in the TE alone phase had sperm counts maintained at less than 3×10^6/mL for 20 wk. Gonadotropin levels of the volunteers were suppressed to 0.4 ± 0.2 IU/L and 0.5 ± 0.2 IU/L in the induction phase, which was stable in the maintenance phase *(351)*. These data not only demonstrated that the daily dose requirements of cetrorelix and Nal-Glu for effective suppression of reproductive functions might be considerably lower than believed in earlier trials, but it also made the development of a depot preparation feasible. In the first clinical trial of a cetrorelix depot preparation, cetrorelix pamoate, was tested in normal and healthy men *(352)*. Volunteers initially received 10 mg cetrorelix/d subcutaneously for 4 d followed by a single intramuscular injection of 60 mg cetrorelix pamoate. Serum testosterone levels remained suppressed for up to 4 wk, whereas in another group of men that had received a placebo depot injection, testosterone values returned to baseline within 2 wk. This study shows for the first time that a GnRH antagonist depot formulation can suppress reproductive hormone secretion in men for several weeks and represents an important step toward the development of a clinically feasible and acceptable mode of GnRH antagonist administration for chronic use.

In conclusion, GnRH antagonists, in combination with testosterone preparations, are highly effective and reversible inhibitors of human testicular function and have a higher potential for endocrine male contraception. However, they must be either easy to use (e.g., oral) or must have a long-lasting depot effect.

CONCLUDING REMARKS AND OUTLOOK

At present, hormonal fertility regulation provides the most promising method for men who wish to control their fertility effectively and reversibly. In this sense the Leydig cell represents a target for hormonal male contraception but the selective suppression of Leydig cell function is not sufficient to accomplish the goal of fertility regulation. Complete suppression of human testicular function requires additional inhibition of FSH release. The chances to achieve azoospermia by hormonal male contraception are higher the lower baseline sperm counts are and the more suppressed the gonadotropins are the less sensitive the AR is.

The present chapter has focussed on aspects of the endocrine regulation of spermatogenesis that are important for the furthermore improvement of hormonal male contraceptives. Effective and reversible regimens for endocrine male contraception have been demonstrated in a multitude of trials that proved the principle: the administration of long-acting testosterone preparations combined with gestagens or GnRH antagonist appear to be capable of lowering sperm counts in men reversibly to azoospermia or at least to very low levels making conception unlikely.

Results from studies performed to date justify development of hormonal contraceptive products for general use. Phase II studies, prerequisite for further development, should target suppression of spermatogenesis as the main parameter. The goals should be azoospermia and a recovery that reaches sperm concentrations of more than 20×10^6 sperm/mL ejaculate.

Long-term safety has to be monitored by postmarketing surveillance. Practical and cheap formulations of the aforementioned compounds will enable their widespread and convenient use and are now to be brought to the market by the pharmaceutical industry.

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30 Clinical Evaluation of Leydig Cell Function

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SUMMARY

Disorders of Leydig Cell function can be primary or secondary to abnormal secretion of LH and FSH. These disorders can be congenital or acquired. The clinical manifestations depend on: (1) location of the defect; (2) age at onset of the disorder; and (3) the nature of associated nonreproductive problems. Because of the critical role of intratesticular testosterone in germ cell maturation, Leydig cell dysfunction often leads to infertility. Testosterone replacement therapy is required for androgen deficient males with primary Leydig cell under-function. Males with hypogonadotropic hypogonadism may be treated with testosterone to normalize serum testosterone levels, but reversal of infertility requires gonadotropin treatment.

Key Words: Androgen; hypogonadism; infertility; LH and FSH; testosterone treatment; testosterone.

HYPOTHALAMIC–PITUITARY–TESTICULAR AXIS

The reproductive hormonal axis in men consists of five main components: (1) the extrahypothalamic central nervous system, (2) the hypothalamus, (3) the pituitary gland, (4) the testis, and (5) the gonadal steroid-sensitive end organs (Fig. 1). Leydig cell function is regulated by the pulsatile secretion of luteinizing hormone (LH) from the pituitary, which in turn is regulated by GnRH from the hypothalamus. In the testis, LH stimulates Leydig to produce androgens. High intratesticular concentrations of testosterone are important in the initiation and maintenance of spermatogenesis. The secreted testicular androgens, testosterone, and dihydrotestosterone (DHT), act on numerous end organs to cause development of male secondary sexual characteristics and to provide negative feedback to inhibit the secretion of gonadotropins. Nonsteroid secretory products of the testis may have regulatory effects on gonadotropins in addition to serving as testicular paracrine factors (*see* Chapters 22–24). Leydig cell dysfunction is defined as primary when the problem lies within the testis and secondary when there is hypothalamic–pituitary dysfunction. Testosterone deficiency in patients with testicular disorders results in elevated serum LH levels and an augmented response of LH to GnRH. If seminiferous tubule dysfunction occurs, serum follicle stimulating hormone (FSH) may also be elevated. In contrast, patients with secondary male hypogonadism have low or low–normal serum LH and FSH, and a decreased response to GnRH stimulation. The episodic secretion of LH and FSH results in considerable short-term variation in the serum concentrations of the two hormones. The peak-and trough pattern of blood levels of gonadotropins is of practical clinical importance in that single measurements of circulating LH may be as much as 50% more or less than mean integrated hormone concentrations.

Testosterone is the principal male sex hormone secreted by the testis, with 5–10 mg produced daily in men *(1)*. The production of testosterone is higher during the day resulting in a diurnal variation in serum testosterone levels. Testosterone may act directly on androgen receptors, or may be converted to DHT or estradiol before asserting its effect through the androgen or estrogen receptors (*see* Chapter 25). This conversion of testosterone to DHT by the enzyme 5α-reductase occurs in many organs, although the skin and prostate are quantitatively the most important source for circulating DHT. DHT is converted peripherally to 3α- and 3β-androstanediol; the 3α-androstanediol circulates in the plasma as 3α-androstanediol glucuronide. The plasma concentrations of this metabolite are believed to be representative of local concentrations of DHT and to provide the best correlation with body hair and beard.

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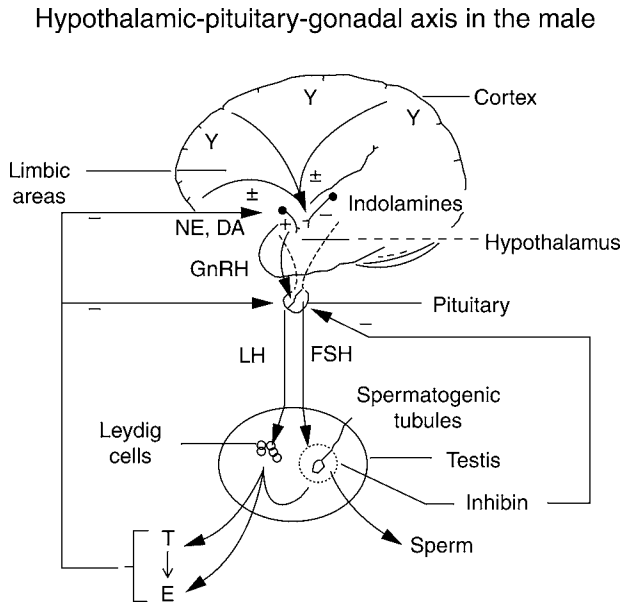


Fig. 1. Hypothalamic–pituitary gonadal axis in man.

3β-androstanediol is an agonistic ligand for the ER-β receptor *(2)* and has been proposed to be an inhibitor of prostate growth. About one-third of the circulating estradiol is secreted directly by the Leydig cell, the reminder is derived mainly from peripheral aromatization of testosterone. Testosterone in the circulation is bound mainly either to a high affinity sex hormone binding globulin (SHBG) or to albumin. The production of SHBG by the liver is increased by estrogens and hyperthyroidism, and decreased by androgens, growth hormone excess, hypothyroidism, and obesity. Only the non-SHBG bound testosterone, including free and albumin-bound testosterone are bioactive (or bioavailable) at the target tissues.

CHANGES IN THE REPRODUCTIVE AXIS WITH AGE

Childhood and Puberty

Serum LH and FSH levels are low at birth but rise for several months after birth and then decline to low and often to undetectable levels by 9–12 mo. Figure 2 presents serum LH, FSH, and testosterone concentrations in boys aged between 2 and 21 yr *(3)*. During early puberty, the mean serum LH levels increase owing to an increase in both pulse amplitude and frequency. In contrast, serum FSH levels increase progressively from prepuberty to midpuberty (4). Beginning at approx age 10–12 yr, there is a much steeper increase in blood testosterone than gonadotropin levels. Figure 3 shows the sleep-related episodic secretion of LH and FSH seen in children at midpuberty *(5,6)*. LH is the primary stimulus for testosterone secretion, whereas both LH and FSH are important determinants of the induction and maintenance of the spermatogenic process. Normal puberty is initiated at the hypothalamic level with increases in secretion of GnRH. Recent data suggest that a kisspeptin-GRP 54 pathway may serve as one of the important hypothalamic initiations of puberty *(7)*. Other factors such as leptin serve as a signal to the hypothalamus indicating that a critical amount of adipose tissue is present for the initiation of pubertal development *(8)*.

Changes in Old Age

Early cross-sectional studies show testicular function decreases in elderly men *(9,10)*. Associated ill health may depress testicular function, but recent longitudinal studies confirmed that lower serum total and especially, free testosterone concentrations are common in healthy elderly men (Fig. 4; refs. *11,12*). Other androgens such as DHT, 3α-androstanediol, and 3α-androstanediol glucuronide also decrease with aging *(11,12)*. In contrast serum estradiol and estrone levels are maintained or increase with age, as a result in part of increased peripheral aromatization of androgen to estrogen *(9)*. Despite wide individual subject variations, both cross-sectional and longitudinal studies show an age-related increase in LH levels after age 30–40 yr. Data from the Massachusetts Male Aging Study estimated the increase as 0.9% per year. Associated increases in FSH suggest that seminiferous tubule degeneration with decreased inhibin production may occur in addition to decreases in Leydig cell function. When coupled with lowered serum testosterone levels, increased LH and FSH provide evidence for a primary defect at a testicular level (Fig. 5) *(11)*. An additional secondary hypothalamic–pituitary defect(s) has been demonstrated in elder men based on the observation that increases in blood gonadotropin levels in androgen-deficient elderly men appears to be blunted when compared to those in younger men with similar degrees of androgen deficiency. Furthermore, there is loss of the diurnal rhythm of testosterone *(13)* suggesting an hypothalamic defect.

CLINICAL PRESENTATIONS OF THE HYPOGONADAL MALE

Age Dependent Manifestations

The clinical manifestation of androgen deficiency depends on the age of onset. During early fetal life androgen deficiency presents with ambiguous genitalia (testicular agenesis, androgen biosynthetic defects, or androgen resistance) in late fetal development; and in

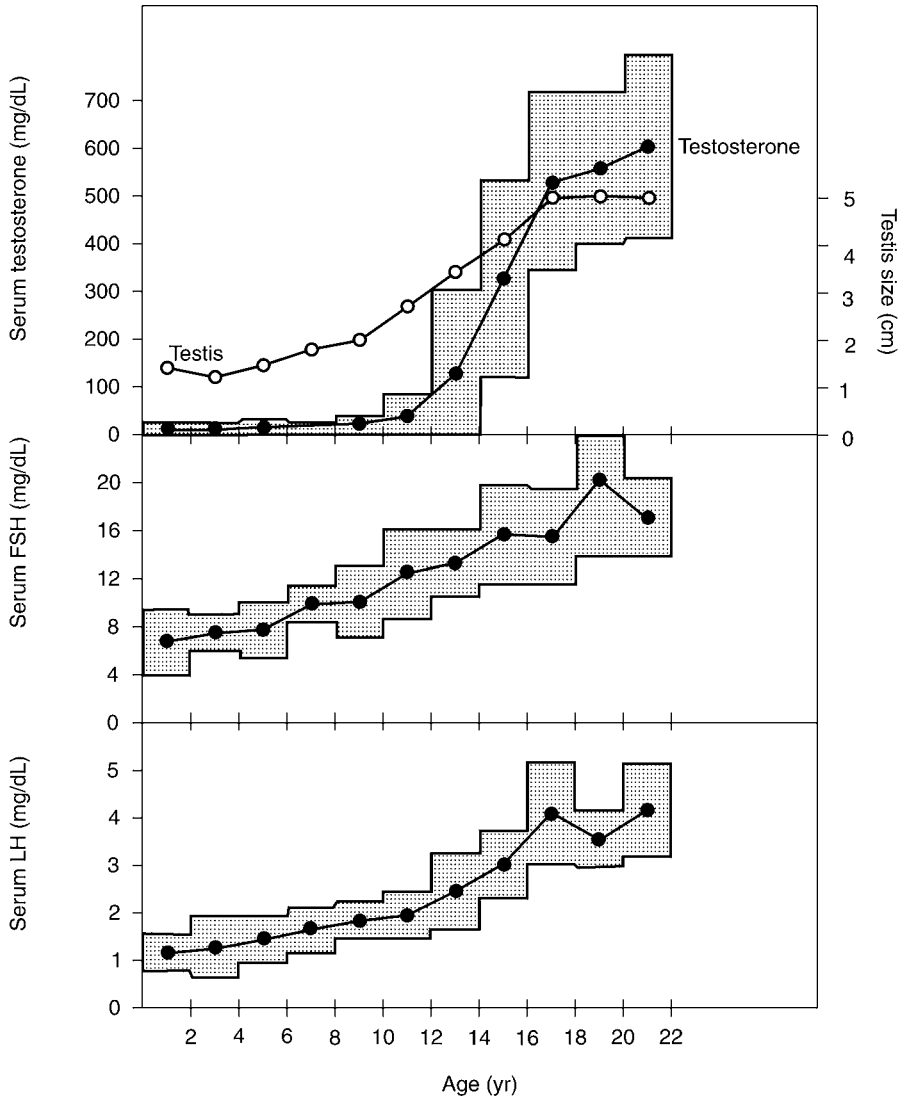


Fig. 2. Cross-sectional data demonstrating the changes in testis size and serum luteinizing hormone, follicle-stimulating hormone, and testosterone levels during sexual maturation in boys. Not shown are the relatively higher luteinizing hormone and follicle-stimulating hormone levels seen in the first year of life. From ref. *3*.

the neonate as micropenis; in the adolescent as delayed pubertal development with eunuchoid features and high pitch voice; and in adulthood as loss of secondary sex characteristics, decreased sexual function, and infertility.

Clinical Features of Leydig Cell Dysfunction

The evaluation of a patient with hypogonadism begins with a detailed and complete history and a physical examination. The medical history should focus on testicular descent, pubertal development, loss of body hair, decrease in shaving frequency, and past and current chronic medical illnesses. In patients presenting with infertility, information should be obtained on previous mumps orchitis, sino-pulmonary complaints, sexually transmitted diseases, genitourinary tract infections, and previous surgical procedures such as vasectomy, orchiectomy, and surgery around the vas deferens. Social history should include tobacco and alcohol intake, exposure to toxic chemicals, hot baths and saunas, irradiation, anabolic steroids, cytotoxic chemotherapy, and drugs that may cause hyperprolactinemia. A detailed sexual history should be obtained including questions on libido, frequency of intercourse and erectile and ejaculatory functions. In addition, the fertility status of the female partner, past investigations of the female partner, and the patient himself should be ascertained.

The physical examination includes a general medical examination. Height, span, and ratio of upper to lower body segments will determine if a patient is eunuchoidal. Androgen deficiency may lead to increased

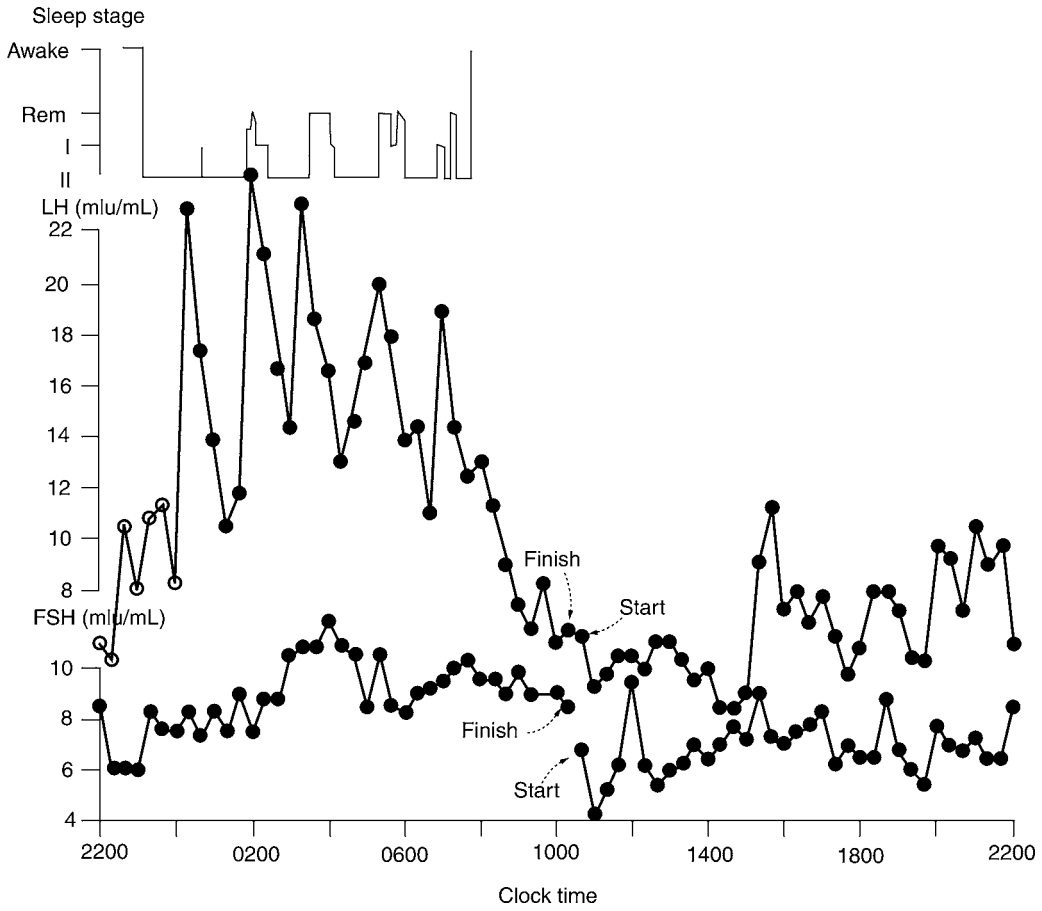


Fig. 3. Plasma luteinizing hormone and follicle-stimulating hormone concentration samples every 20 min for 24 h in a normal pubertal child. Gonadotropin levels are elevated during sleep. From ref. *5*.

body fat and decreased muscle mass. Obesity leads to lower testosterone levels. This is in part because of decrease in sex hormone binding protein levels and in some individuals with more severe obesity, a decrease in testosterone secretion. Loss of pubic, axillary, and facial hair, decreased acne and oiliness of skin, and fine facial wrinkling are features suggestive of androgen deficiency. Gynecomastia may be present when there is a decreased androgen to estrogen ratio. During the physical examination, the stage of development (Tanner's classification) of the gonads and phallus is ascertained. Examination of the scrotum should include palpation of the vas and epididymis and the identification of other scrotal abnormalities such as varicocele, hydrocele, and hernia. Testis size can be measured by the Prader orchidometer consisting of a series of plastic ellipsoids with a volume from 1 to 35 mL. A testis volume of less than 15 mL in an adult Caucasian man is regarded as small; testicular size is slightly less in healthy Asian men. A decreased testis volume usually indicates a decreased mass of the seminiferous tubules as they account for over 80% of the mass of the adult testes.

LABORATORY TESTS FOR MALE HYPOGONADISM

In most circumstances, diagnosis is made from the assessment of serum testosterone concentration in the morning. Serum LH and FSH measurements may help to distinguish primary (testicular) from secondary hypogonadism (hypothalamic–pituitary) disorders.

Testosterone (Total and Free or Bioavailable)

Testosterone concentrations are measured by radioimmunoassays, immunometric assays, or immunofluorometric assays. Testosterone secretion has a circadian rhythm in man with higher levels in the morning than evening. Because reference ranges are based on morning values, blood samples for testosterone measurements should be drawn in the morning. Automated assays for testosterone are frequently utilized by clinical

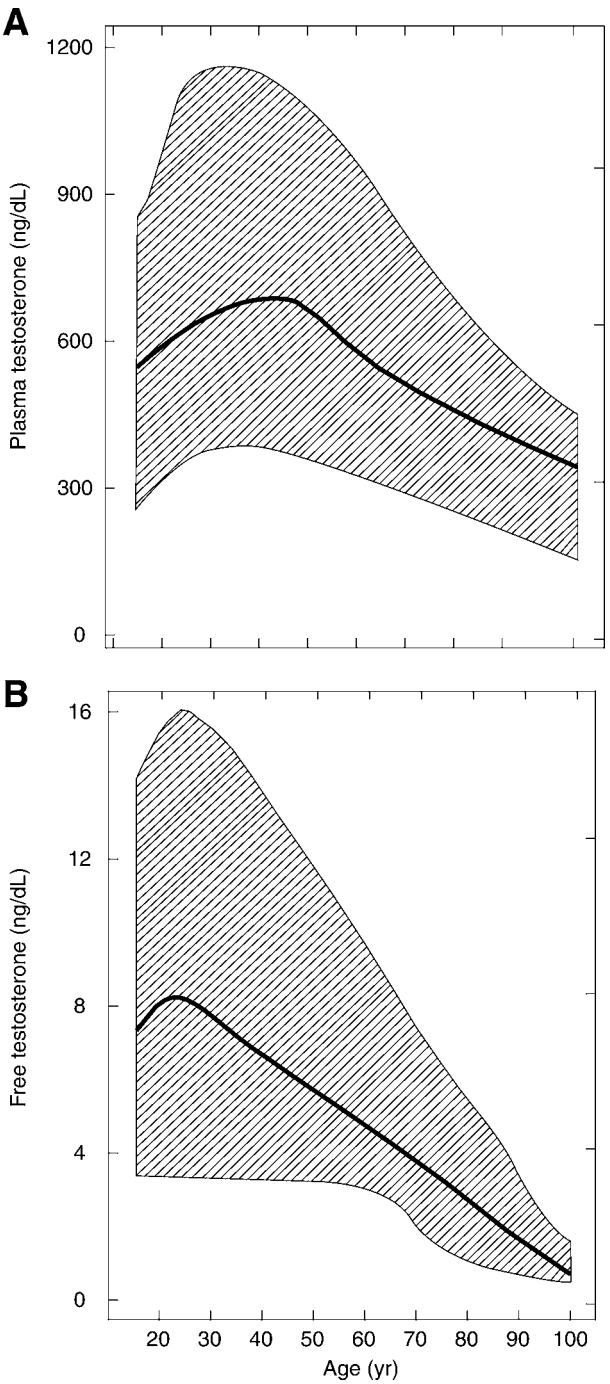


Fig. 4. Plasma total and free testosterone in a group of men of differing ages. Both total and free testosterone decline with age, but the decrease in free testosterone is much more in the older man from ref. *9*.

laboratories. In general, these assays have variable and often poor levels of accuracy in the female, children, and severely hypogonadal ranges. Liquid chromatography, tandem mass spectroscopy is the emerging technology for most precise and accurate testosterone measurements *(14,15)*.

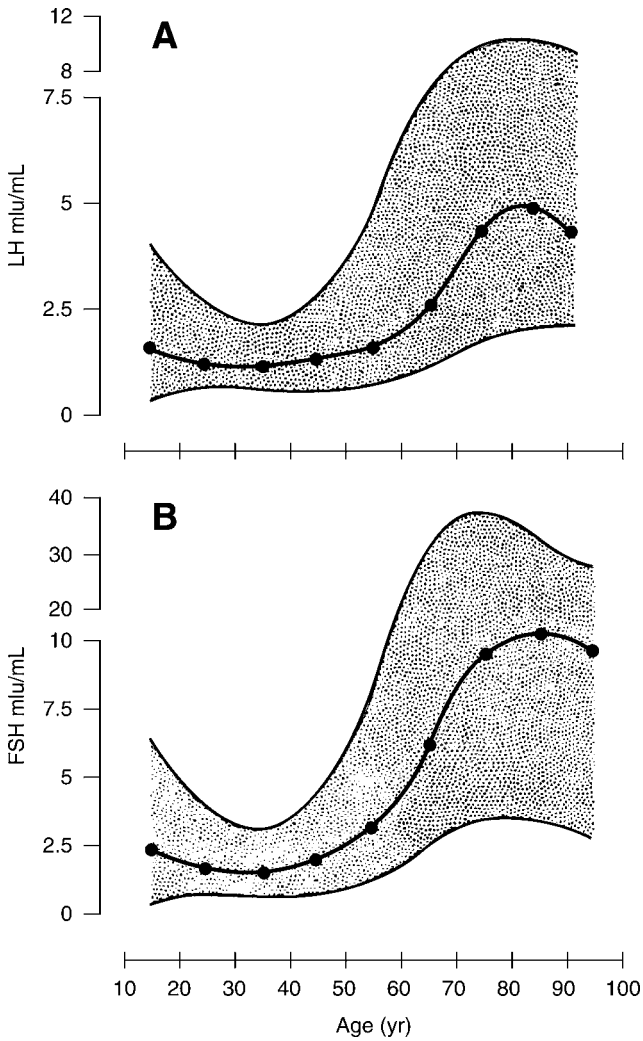


Fig. 5. Serum-luteinizing hormone and follicle-stimulating hormone in men of increasing age. From ref. *9*.

In most instances, measurement of total serum testosterone will identify patients with androgen deficiency. However, because testosterone is bound to SHBG in the plasma, changes in SHBG concentrations will lead to changes in total testosterone concentrations. In disorders with abnormal SHBG concentrations, the measurement of total testosterone may be misleading. For example, an overweight patient may have low total testosterone concentrations reflecting the low SHBG concentrations associated with obesity. To separate true hypogonadism from binding protein problems, it may be necessary to determine the free or bioavailable testosterone concentrations. Free testosterone concentrations are usually measured after equilibrium dialysis of a serum sample. About 2% of testosterone in blood is free, the rest is bound to SHBG (30%) or to albumin and other proteins (68%).

The non-SHBG bound testosterone, (i.e., free and albumin bound) is the bioavailable portion of circulating testosterone. Bioavailable testosterone can be estimated in the serum by radioimmunoassays, after removal of SHBG by ammonium sulfate or Concanavalin A-Sepharose. Salivary testosterone can also be used as an indicator of free testosterone as SHBG and other proteins are present in very low concentrations in the saliva.

Other Androgen Metabolites

Generally, the measurement of plasma DHT is not useful in the evaluation of testicular disorders other than 5α-reductase deficiency. In this disorder, measurement of serum DHT and testosterone will show an abnormally high testosterone to DHT ratio, especially after administration of human chorionic gonadotropins (hCG). Estradiol measurements are usually not necessary in the assessment of male reproductive disorders. Estradiol concentrations are elevated in patients with androgen resistance, estrogen secreting neoplasms, Klinefelter's syndrome (KS), and hypogonadism associated with chronic liver disease.

Luteinizing Hormone and Follicle-Stimulating Hormone

Measurements of serum LH and FSH are important in classifying the anatomical level of the defect in hypogonadal patients (Fig. 1). As both gonadotropins are secreted in a pulsatile pattern, collection of three samples at 15–20 min apart may give more accurate assessment of the mean LH and FSH concentrations. The multiple sampling is not routinely used in the clinic. Primary gonadal defects are characterized by low testosterone and high LH and FSH concentrations whereas hypothalamic or pituitary disorders have low testosterone, LH, and FSH concentrations. These sensitive immunofluorometric gonadotropin assays allow the clinician to distinguish the low gonadotropin concentrations commonly observed in hypogonadotropic hypogonadism, delayed puberty, and after gonadotropin releasing hormone (GnRH) analog treatment. Unlike the serum testosterone assays, measurement of gonadotropins showed less variation between laboratories using the automatic immunoassays *(16)*.

Other Pituitary Hormones

Serum prolactin concentrations should be measured in patients with low testosterone and normal to low FSH and LH concentrations to exclude hyperprolactinemia. Measurements of the α-subunits of the gonadotropins may be useful in patients with pituitary tumors with or without hypogonadism. Many pituitary tumors previously believed to be nonfunctioning, secrete large amounts of α- and β-subunits of LH and FSH. In patients with germinomas, teratomas, and chorioepitheliomas, β-hCG subunits may also be secreted and can serve as a tumor marker.

DynamicTests of the Hypothalamic–Pituitary–Testicular Axis

Before the development of sensitive assays for the gonadotropins, dynamic tests to evaluate the hypothalamic–pituitary axis were developed. Now with more sensitive and specific gonadotropin assays, these dynamic tests are reserved for occasional and unusual diagnostic problems. The GnRH test (administered as a 100 μg bolus to adults or 50 μg/m^2 to children) should allow hypothalamic disorders to be distinguished from pituitary disorders. However, the gonadotropin response to a single dose of GnRH is frequently suppressed in patients with hypothalamic disorders. Clinically, GnRH test are used in pediatric endocrinology for the diagnosis of delayed or precocious puberty. Leydig cell function can be stimulated by a single injection of (hCG, 2000–5000 IU), resulting in peak increases in plasma testosterone concentrations after 72–96 h.

The hCG test is useful in infants or children with cryptorchidism because a rise in testosterone in response to hCG indicates presence of the testes and excludes anorchia. Multiple and frequent sampling for LH and FSH concentrations has been used to delineate the defect in GnRH secretion in patients with idiopathic hypogonadotropic hypogonadism and aging men. Disorders of LH pulsatility including absence of pulse, sleep-entrained pulses, and decreased frequency of amplitude of pulses have been defined. Sleep-entrained pulsatile secretion of LH is a hallmark of the onset of puberty and can be used to distinguish patients with early puberty from those with hypogonadotropic hypogonadism. However, multiple blood samples have to be taken at 10 min intervals for a minimum of 8 h to yield meaningful analyses of the pulsatile secretion of the gonadotropins. Because of the frequency and intensity of sampling, these investigative procedures are used mainly in clinical research studies.

Endocrine Tests in the Evaluation of Ambiguous Genitalia

The diagnosis of a neonate presenting with ambiguous genitalia is beyond the scope of this chapter. In brief, the chromosomal sex should be determined. Testosterone biosynthetic defects are diagnosed by the measurement of the precursors of testosterone. Male pseudohermaphroditism occurs in CYP17 (17 α-hydroxylase and 17, 20 lyase deficiency), and

17 β-hydroxysteroid dehydrogenase deficiency (*see* Chapter 10). The laboratory diagnosis of androgen resistance syndrome is made by measuring androgen receptor binding and function in genital fibroblast cultures. In patients with this disorder, androgen receptor binding may be low, undetectable or unstable or abnormal, androgen receptors and postreceptor abnormalities may be present. Single point mutations, multiple mutations, deletion, or premature stop codon defects have been identified to cause absent, quantitatively, or qualitatively abnormal androgen receptors *(14,15,17–19)*. 5α-reductase deficiency can be diagnosed by the measurement of the ratio of testosterone to DHT in plasma before and after hCG stimulation. In Leydig cell hypoplasia or aplasia, the Leydig cells are resistant to LH and hCG. Serum testosterone concentrations are low and do not respond to exogenously administered hCG; the testes are small and atrophic, and testicular biopsy shows hypoplasia or absence of Leydig cells. In the vanishing testes syndrome, endocrine tests yield the same findings as in Leydig cell hypoplasia. However, imaging by ultrasound, CT scan, or magnetic resonance fails to demonstrate the presence of testes. Recently, assays of Mullerian Inhibiting Factor have been found to be very low or undetectable in patients with anorchia.

Semen Analyses

In men with Leydig cell dysfunction and male hypogonadism, impaired spermatogenesis is very common. In general determination of semen volume, sperm count, motility, and morphology would be sufficient for the investigation of a patient with severe oligozoospermia (fewer than 5 million spermatozoa per ml semen) or asthenozoospermia (fewer than 10% motile) or teratozoospermia (fewer than 10% normal). In patients with normal or moderate impairment of semen parameters, further evaluation with specialized tests might be helpful to delineate specific defects of sperm function. Because of the marked inherent variability of semen parameters, at least two semen analyses at 1–2 wk intervals should be assessed in the laboratory. The semen sample should be collected by masturbation. The procedures and methods for routine semen analyses should follow the World Health Organization Laboratory Manual for Human Semen and Sperm Cervical Mucus Interaction *(20)*. A normal semen sample should have a sperm concentration of more than 20 million/mL or total sperm count of more than 40 million and 50% or more spermatozoa with progressive motility and over 10 sperm with normal morphology using the strict criteria. More recent studies suggest that lower sperm concentration of around 10–15 million/mL may be associated with normal fertility *(21,22)*.

DISORDERS OF MALE REPRODUCTION AND APPROACH TO DIAGNOSIS

Approach to Male Hypogonadism

Hypogonadism refers to patients that are deficient in androgen secretion and/or sperm production. Most androgen deficient patients are infertile, whereas most infertile patients have serum testosterone levels within the normal range. Usually, patients with hypogonadism are classified as either primary testicular dysfunction or hypothalamic–pituitary disorders (Table 1). Combinations of the two may occur as in aging, hepatic cirrhosis, HIV infections, severe illnesses, and sickle cell disease. With the basal serum concentrations of FSH, LH, and testosterone and some of the laboratory tests discussed in the previous section, a clinician can usually determine the cause of hypogonadism following the algorithm shown in Fig. 6 *(23)*. Low concentrations of testosterone, FSH, and LH are indicative of hypogonadotropic hypogonadism. Prepubertal onset is usually indicative of a congenital defect such as Kallman's syndrome. Prolactin concentrations should be checked to exclude hyperprolactinemia. Anterior pituitary hormone function should be assessed and a magnetic resonance imaging (MRI) scan performed to exclude hypothalamic–pituitary tumors. Elevated FSH and LH concentrations in the presence of low testosterone indicate primary testicular failure. A karyotype could be performed to exclude KS. Isolated elevations of FSH in the presence of normal LH and testosterone indicate isolated Sertoli dysfunction and germinal epithelium damage as is commonly seen in azoospermic infertile men. Elevated LH concentrations in the presence of elevated testosterone and estradiol levels suggest androgen resistance; genital skin fibroblast studies will define the abnormality of the androgen receptor. If these hormone measurements are normal and the patient has clinical symptoms or signs of hypogonadism, then a free or bioavailable testosterone can be measured to exclude patients with low free testosterone and elevated SHBG as in elderly men.

Common Causes of Hypogonadism

In this section only a few of the common diseases causing male hypogonadism will be discussed. For details of the less common causes of hypogonadism the reader is referred to a standard text of endocrinology *(24)*.

Primary Gonadal Failure

The list of specific etiological causes is long (Table 1) but often definable by careful history, physical examination, and laboratory tests. KS is the most common

Table 1
Common Causes of Male Hypogonadism

Hypothalamic–pituitary disorders (secondary Leydig Cell Dysfunction)
Idiopathic GnRH deficiency, Kallmann's Syndrome, Prader Willi, Laurence-Moon-Biedl syndromes, multiple hypothalamic deficiency, pituitary hypoplasia
Trauma, postsurgical, postirradiation
Tumor (adenoma, craniopharyngioma, others)
Vascular (pituitary infarction, carotid aneurysm)
Infiltrative (sarcoidosis, histiocytosis, tuberculosis, fungal infection, hemochromatosis)
Systemic illness, malnutrition, anorexia nervosa, obesity, diabetes mellitus
Autoimmune hypophysitis
Drugs (drug-induced hyperprolactinemia, sex steroids)
Testicular disorders (Primary Leydig Cell Dysfunction)
Chromosomal (Klinefelter's syndrome and variants, XX male gonadal dysgenesis)
Defects in androgen biosynthesis
Orchitis (mumps, other viral, HIV, leprosy)
Autoimmune orchitis
Cryptorchidism
Myotonia dystrophica,
Toxins (alcohol, opiates, fungicides, insecticides, heavy metals, cotton seed oil)
Drugs (cytotoxic drugs, ketoconazole, cimetidine, spironolactone)
Systemic illness (uremia, liver failure)
End organ disorder (impaired androgen action)
Androgen receptor defects
Post receptor transduction abnormalities
5α-reductase deficiency
Spinobulbar muscular atrophy (Kennedy's disease)
Others
Obesity
Diabetes Mellitus Type 2
Hemochromatosis
HIV disease

form of congenital primary male hypogonadism (1 in 500–1000 male births) and is characterized by small testes, androgen deficiency, azoospermia, bilateral gynecomastia, and increased gonadotropins. In most cases, the chromosomal anomaly arises from nondisjunction of a maternal or paternal sex chromosome during the first meiotic division. Other nonreproductive defects occur including cognitive disabilities; behavioral dysfunction, abnormalities of tooth structure, and atypical finding of relatively longer lower extremities than upper extremities. The phenotypic manifestations of KS are most classical in men with a 47 XXY karyotype. Some men with the clinical picture of KS have a mosaic pattern, XY/XXY. Less common situations are XXY, XXXY, and mosaics.

Diagnosis of KS before to puberty is often difficult, although learning disabilities, attention deficits, and behavioral dysfunction may raise suspicion of KS. The testes are usually small in the neonatal period and fail to increase in size at puberty and remain less than 3 mL in volume. LH and FSH levels tend to be normal before puberty but rise more than the normal range at the age of physiological pubertal increases in reproductive hormones. Gynecomastia occurs to varying degrees in the postpubertal period probably because of decreased ratios of testosterone to estradiol. In KS, muscle mass is usually diminished and strength decreased. Beard and body hair is reflective of the testosterone levels in the patient. The prostate is small and does not increase in size until androgen treatment is begun. Serum testosterone levels are usually low or low normal with free testosterone levels more predictably decreased owing to increased SHBG levels. In many instances, a temporary state of compensated hypogonadism may be present (elevated serum LH and FSH with normal serum testosterone) but testosterone levels fall as the patient ages. Serum LH and FSH levels are uniformly elevated in adult KS patients. Azoospermic infertility is the rule in KS, with typical testicular biopsies revealing Leydig cell hyperplasia, loss of germ cells, sclerosis of the germ cell compartment, and thickened tubule basement membranes. Mosaic forms may have some degree of

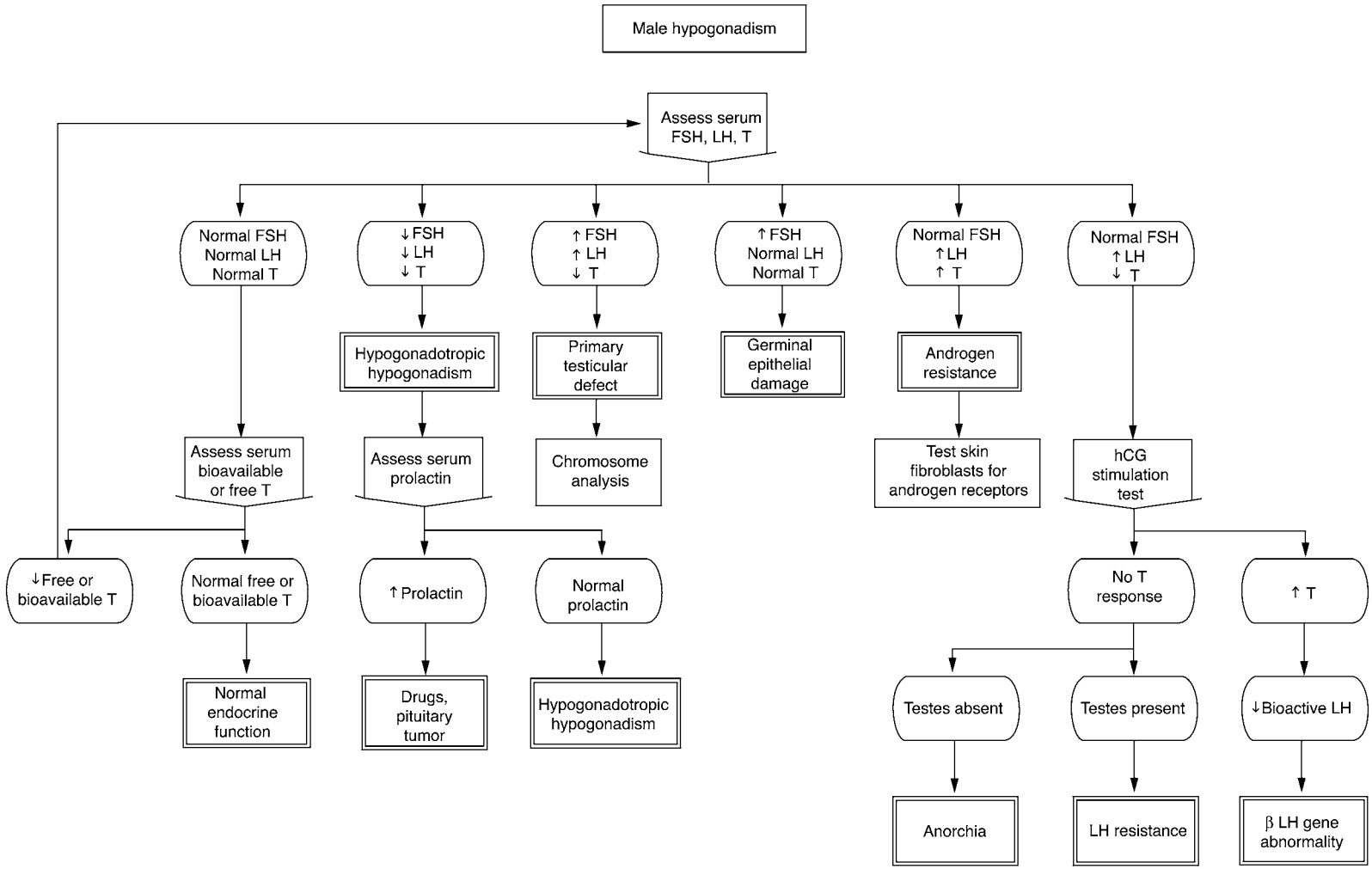


Fig. 6. Diagnostic approach to a patient with male hypogonadism. FSH, follicle stimulating hormone; LH, luteinizing hormone; T, testosterone; hCG, human chorionic gonadotropin; MIF, Mullerian inhibiting factor. From ref. *23*.

immature germ cells on biopsy but almost all are azoospermic *(25)*.

The reasons for the cognitive dysfunction in KS are not known but the selective learning (dyslexia) and behavioral difficulties suggest a CNS integrative disorder reminiscent of other frontal-temporal lobe disorders). Neuroimaging by MRI showed anomalous cerebral laterality and significant reduction in the total left temporal gray matter volume in KS adults *(25–27)*. Despite typical histories of poor school advancement and work habits, many KS patients test well on global IQ testing with some scoring in the superior range. The reasons why extra X chromosomes produce the clinical spectrum of events are unknown but may be related to over expression of genes that are not susceptible to inactivation in the supernumerary X chromosomes. The hypogonadal manifestations in patients with KS respond favorably to testosterone replacement, whereas the cognitive dysfunction seems to be immutable or at best variable in its response. Several mouse models of XXY aneuploidy have been developed *(28,29)*, which show reproductive and cognitive-deficits. These models may allow more insight into the molecular basis of the various phenotypic manifestations of KS. Studies of phenotype (social and physical defects) and genotype (length of CAGn of the androgen receptor) showed that XXY men (KS) with longer CAGn had more clinical manifestations of testosterone deficiency and were less responsive to testosterone therapy *(30)*. Myotonic Dystrophy presents later in life (after age 30) and is associated with testicular atrophy, decreased fertility, and hypergonadotropic hypogonadism. Autoimmune testicular failure owing to antibodies against the microsomal fraction of the Leydig cells may occur either as an isolated disorder or as part of a multiglandular disorder involving the thyroid, pituitary, adrenals, pancreas, and other organs.

Mumps orchitis following puberty is associated with clinical orchitis in 25% of cases. About 60% of men with clinically induced mumps orchitis will become infertile. Cryptorchidism is high at birth (10%) but most will descend to the appropriate scrotal location in early childhood. The incidence of bilateral undescended testes is 0.3–0.4% following puberty. Cryptorchidism is associated with infertility most likely owing to the dysgenetic testis. Androgen deficiency (Leydig cell dysfunction) is less common but does occur. Cryptorchidism should be treated by bringing the testes into the scrotum in early childhood (before age 5), thus decreasing the chances of permanent infertility and the testicular malignancies (8%) associated with abdominal testes.

Testicular irradiation as a result of accidental exposure in treatment of associated malignant disease will produce testicular damage. A dose as low as 15 rads will cause transient decreases in the sperm count; 50 rads exposure may cause azoospermia. After 500 rads, the infertility is usually irreversible. Chemotherapy for malignant disorders may cause irreversible germ cell damage. Toxins and Environmental Endocrine Disruptors may also directly damage the testes. Many agents such as fungicides and insecticides (e.g., DBCP and Vinclozolin), heavy metals (lead, cadmium), and cottonseed oil (gossypol) cause damage to the germ cells. Usually, Leydig cell function is relatively less susceptible to most chemotherapeutic drugs and toxins. Medications may interfere with testosterone biosynthesis (e.g., ketoconazole and spironolactone) thus producing Leydig cell dysfunction. Trauma, torsion of the testes and vascular injury may produce hypogonadism and infertility.

Androgen End Organ Failure

Decreased end organ responsiveness to circulating testosterone results in clinical phenotypes mimicking Leydig cell dysfunction but serum testosterone levels are normal or elevated.

Androgens Receptor Defects have been reported producing a spectrum of clinical manifestations from "complete" forms (testicular feminization), to "incomplete" forms (Reifenstein's syndrome), to "minimal" forms (hypospadias). In testicular feminization, there is no binding of T and DHT to a mutant AR. The patients are phenotypically female with normal appearing breasts and external genitalia, but have blind vaginal pouches; the absence of the uterus leads to amenorrhea. The testes are present in the labia canal or intra-abdominal. Serum testosterone levels are normal or elevated and serum LH and FSH levels might be elevated owing to lack of testosterone feedback on the hypothalamic–pituitary axis. Breast development and female fat distribution reflects increased estradiol levels and unimpeded estrogen effects. The testes should be removed because of increased risk of malignancy and estrogen replacement therapy will be needed as these patients are to be treated as though they were hypogonadal women. Reifenstein's syndrome is a form of partial androgen resistance. Decreased receptor number, decreased receptor stability, and post receptor response defects are responsible for the hypogonadal state and varying degrees of defective external genitalia differentiation (including bifid scrotum and hypospadias) owing to incomplete midline fusion of the urethra and labial folds. Gynecomastia frequently occurs at puberty when

LH, testosterone, and estradiol levels rise. More subtle defects limited to hypospadias (abnormal opening at the urethra on the ventral shaft of the penis or perineum) and/or impaired spermatogenesis have also been described. Treatment of incomplete AR deficiency with high doses of testosterone has been only partially successful.

Androgen receptor polymorphisms involving differences in the length of CAG repeats of exon 1 of the androgen receptor is inversely associated with androgen action. There is a racial distribution with Asian men having longer CAGn and African American men shorter repeats *(31)*. Patients with Kennedy's syndrome (Spinobulbar Muscular Atrophy) have expanded CAG repeats of the androgen receptor, resulting in lower transactivation of the androgen receptor and clinical features of androgen resistance *(32)*.

5α-reductase deficiency is an experiment of nature to illustrate differential testosterone and DHT action. It is marked by diminished levels of the enzyme 5α-reductase 2 responsible for conversions of testosterone to DHT *(33–36)*. As DHT is required in males for the normal development of the external genitalia, growth of the phallus and prostate, these patients have severe pseudohermaphroditism at birth. Because the defect is incomplete, the patients undergo partial masculinization owing to the high levels of testosterone secreted at puberty leading to increases in muscle mass, decreases in body fat and phallic growth, and small prostate. Hypospadias, small testes, and infertility are present. LH, FSH, and testosterone levels are normal in these patients but the ratio of DHT/T is decidedly low. Replacement with DHT is the treatment of choice if the patient has been assigned a male gender.

Hypogonadotropic Hypogonadism

Hypogonadotropic hypogonadism (HH) represents a deficiency in secretion of gonadotropins (LH and FSH) because of an intrinsic or functional abnormality in the hypothalamus or pituitary gland. Such disorders result in secondary Leydig cell dysfunction. As many patients with idiopathic HH (IHH) have a congenital deficiency in GnRH secretion, the manifestations include a small phallus, failure to undergo secondary sexual development at the time of puberty, diminished sexual drive (libido), and decreased metabolic effects of testosterone (decreased muscle and bone mass) as adults. Acquired loss of gonadotropin secretion such as occuring after trauma, pituitary tumors, and hypothalamic or pituitary inflammatory disease might be present owing to the local effects of the CNS disorder (i.e., visual field impairment, hypopituitarism, headaches), or if clinically indistinguishable from primary gonadal failure. In the latter case, suspicion of a central defect (hypothalamic hypogonadism) comes from the laboratory pattern of low serum testosterone and low or inappropriately normal serum LH and FSH levels. The site of the hypothalamic or pituitary lesion should be localized, if possible, by MRI. A serum prolactin must be measured to exclude the presence of hyperprolactinemia owing to a prolactin secreting pituitary adenoma. The distinction between central and testicular causes of hypogonadism is important because secondary hypogonadism might imply: a progressive and/or specifically treatable disorder; the unintended effect of drugs that inhibit the hypothalamic–pituitary axis, such as tranquilizers, antidepressants, and estrogens; systemic illness; malnutrition or anorexia nervosa. The infertility associated with HH may be treatable with gonadotropin or GnRH replacement therapy.

Kallmann's syndrome is characterized by delayed or arrested sexual development and anosmia first described in 1944 *(37)*. The primary defect in these patients and those with idiopathic HH is hypothalamic resulting in impaired gonadotropin secretion. The incidence of this disorder is approx 1 in 10,000 male births. Although, anosmia/hyposmia are the most well-known, Kallmann's syndrome is associated with color blindness, cleft lip and palate, cranial nerve defects (including eighth nerve deafness), horseshoe-shaped kidneys, cryptorchidism, and optic atrophy. Abnormalities of the gonadotropin releasing hormone receptor (GnRH-R) explain some of the defects of IHH. Mutation of the Kiss-1 derived peptide GPR 54 have recently been identified in patients with IHH *(7)*. IHH associated with impaired olfactory function may be caused by mutations of the X chromosomal Kal 1 gene (encoding anosmin) or the fibroblast growth factor (FGFR1) both leading to agenesis of olfactory and GnRH secretory neurons *(38)*. IHH occurs both in sporadic (nonfamilial) and familial forms, the majority of cases are sporadic with no differences in the clinical presentations of the two subgroups. Those with the most severe deficiency may present with complete absence of pubertal development and sexual infantilism, and azoospermia. At least 10% of the patients have partial GnRH deficiency (isolated FSH or LH deficiency "Fertile Eunuch syndrome") and varying degrees of delay in sexual development in proportion to the severity of gonadotropin deficiency.

Acquired hypogonadotropic disorders—anorexia nervosa and weight loss are examples of functional defects resulting in low serum testosterone levels. Predominantly a disorder of adolescent girls, anorexia

nervosa is characterized by excessive weight loss as a result of voluntary dietary restriction. Occasionally, the disorder is seen in men but usually implies a more severe variant of the psychiatric disorder. Strenuous exercise has minimal effects on testicular function in men and contrasts highly with the well-known reproduction dysfunctioning in female long distance runners and dancers. Severe stress and systemic illnesses will lower gonadotropin and testosterone levels. Many severe chronic systemic illnesses may result in hypogonadism. Chronic hepatic and renal disease, cancer, diabetes mellitus, and severe infections are commonly associated with low testosterone levels.

Other Cause of Hypogonadism

Diabetes mellitus and obesity are risks factors for low testosterone levels *(39–42)*. The degree of low testosterone seems to correlate with the increase of blood sugar (hemoglobin A1C levels) and the severity of obesity. Hemochromatosis is associated with male hypogonadism, usually because of deposits of iron in the hypothalamus-pituitary regions. HIV infection is often associated with hypogonadism. Both gonadal and hypothalamic infection with the HIV virus, infection by other organisms, stress, malnutrition, and malignancies may all coexist.

ANDROGEN REPLACEMENT THERAPY

Testosterone Replacement Therapy

The indications for androgen treatment are shown in Table 2 *(43,44)*. The primary use of androgens is to treat male hypogonadism. Primary Leydig cell failure must be treated with androgens to relieve their clinical symptoms and signs. Response to androgen replacement therapy is monitored mainly by improvement in the clinical features of hypogonadism. Improvement in sexual function, frequency of shaving, secondary sexual characteristics, and general well-being occur rapidly after the initiation of treatment. Androgen treatment does not reverse their infertility. Nadir and peak testosterone levels are monitored during the start of therapy and in patients who do not show adequate clinical response. The goal is to maintain the serum testosterone levels in the mid normal range. Recent data have shown that dose response relationships occur between the dose of testosterone administered and the serum levels attained, and the improvement in muscle mass and strength and bone mass, and the decrease in fat mass, whereas a threshold effect applies to sexual function, i.e., when serum testosterone reaches the low-normal range, sexual function appears to be restored to pretreatment levels *(45,46)*. Secondary Leydig cell dysfunction can also be corrected by testosterone replacement therapy. When patients with HH desire a pregnancy, the testes must be stimulated with LH (or hCG) and FSH. Earlier treatment with testosterone does not jeopardize the chances of fertility in these patients with HH.

Table 2
Indications for Androgen Therapy

Definite	Male hypogonadism
Probable or possible	Micropenis in children
	Constitutional delayed puberty
	Aging men (with evidence of androgen deficiency)
	Male Contraception
	Hereditary angioneurotic edema
Dubious or controversial	Improvement of nitrogen balance in nonandrogen-deficient catabolic state
	Improvement of libido in women with hypoactive sexual disorder
Not indicated	Anemia associated with renal failure
	Improvement of muscle strength and endurance in athletes body builders

Other uses of androgens are in young children with micropenis where a short course of low-dose androgen therapy is often tried. In adolescent boys with constitutional delay of puberty in whom the psychological effects of delayed puberty are significant, short-term treatment with testosterone for 3–4 mo may be indicated. As discussed previously, aging is associated with low total and free testosterone levels, which frequently may be associated with symptoms such as a decrease in sexual function. It has not been proved that androgen therapy in older men with borderline or normal range testosterone levels will improve sexual function, prevent bone and muscle loss, or improve the quality of life of aging men *(47)*. Replacement of testosterone is frequently administered to symptomatic older men with definitely low serum testosterone levels. The possible beneficial effect of androgens must be balanced against the possible adverse effects on lipids, prostate, and sleep-related breathing disorders *(48)*.

Testosterone has been given alone or in conjunction with other progestins and GnRH analogues as experimental male contraceptives. Recent data indicate that pharmacological doses of testosterone will successfully suppress sperm counts to levels incompatible with fertility. In these regimens, androgens function both to suppress sperm production by inhibiting gonadotropins

and to replace endogenous androgen levels. The dosage of testosterone used in successful male contraceptive trials is higher than replacement and long term data on possible adverse effects on the prostate and cardiovascular system are not yet available *(49,50)*.

In hereditary angioneurotic edema, anabolic steroids have been used to prevent attacks. These anabolic steroids increase the synthesis of complement 1 inhibitor, which is deficient in these patients. The role of androgens in the treatment of hematological or renal disorders has been superseded by newer more specifically targeted treatments.

Metabolic Effects of Androgens

The anabolic effects of testosterone on bone and muscle are of prime importance in maintaining normal strength and vigor as well as prevention of osteoporosis and fractures. Testosterone, in physiological doses, will reverse the muscle wasting seen in hypogonadal men and pharmacological doses will enhance these parameters in normal men *(45,46,51)*. Castrate and other hypogonadal men lose bone mineral density at an accelerated rate and are prone to fractures, and treatment will reverse the process *(52,53)*. It is unclear if the effects on retention of bone mineral density are moderated through conversion of testosterone to estradiol, directly through testosterone on the androgen receptor or both. Recent reports of severe osteoporosis in patients with normal serum testosterone levels and estrogen receptor, or aromatase defects suggest that aromatization of testosterone to estradiol may be a critical step in androgen action on bone *(54–56)*. Testosterone deficiency will lead to decreased blood cell mass probably secondary to decreased erythropoietin production and by loss of direct effects of androgens on the bone marrow. Pharmacological doses of testosterone will increase hematocrit, hemoglobin levels, and white blood cell concentrations.

Androgen Delivery Systems

Current androgen replacement methods included testosterone buccal tablets, oral testosterone undecanoate (not available in the United States), testosterone transdermal patches and gels, and testosterone esters injections. The choice of the different preparation depends on the patients' disease, their preference, and the physician's experience with the preparations *(43)*.

Androgens in Athletes

The pattern of androgen use by athletes involves the intermittent and cyclical administration of pharmacological doses of a combination of oral and parenteral agents *(57,58)*. These unprescribed androgens may include huge doses of drugs, including veterinary agents that either are potentially toxic or have not been tested in humans. In a recent placebo controlled study, the effect on testosterone muscle size and strength was progressive, indicating that the performance enhancing effects of androgens are dose-related *(45)*. Some athletic trainers and physicians have argued that even small changes in performance justify the use of these agents by high-performance, competitive athletes. The policies of all international and US athletic regulatory agencies are unambiguously opposed to "doping" with androgens or other medicines to improve performance. The long-term abuse of supraphysiological doses of androgens in men may lead to gynecomastia, hepatic toxicity (caused by 17-alkylated androgens), polycythemia, lipid changes (lowering of high-density cholesterol), and suppression of spermatogenesis.

Potential Adverse Effects of Androgen Treatment

In general, testosterone and its esters have fewer side effects than the synthetic 17-alkylated androgens *(44,59,60)*. Acne and increased oiliness of skin are frequently experienced by patients at the initiation of androgen supplementation. Because testosterone is metabolized to estradiol, gynecomastia may develop. The gynecomastia is often mild and treatment is usually unnecessary. Most patients gain weight when administered androgens. The weight gain is owing to water retention, increased blood volume, and increased lean body mass. Patients given exogenous androgen therapy have reversible suppression of spermatogenesis and a decrease in testicular size (Liu, et al: Lancet, 2006, in press). The decrease in sperm production and seminiferous tubule volume are consequences of the suppression of GnRH, LH, and FSH. Androgens promote premature epiphysial closure of the long bones in children and will result in reduced ultimate height. For these reasons, androgens should not be used in prepubertal boys except for specific indications discussed previously.

Changes in liver function and hepatic disorders are not observed with testosterone or its esters but may be seen with 17-alkylated androgens. The 17-alkylated androgens are also associated with decreases in high-density lipoprotein cholesterol and apolipoprotein A-I and A-II levels and increases in low-density lipoprotein cholesterol and apolipoprotein; these changes in lipid profile are risk factors for coronary atherosclerosis. Changes in lipid profile occur to a considerably lower extent with testosterone esters, provided physiological

replacement is attained. Androgens cause small increases in hemoglobin, hematocrit, and total red cell count when administered to normal or hypogonadal men. Androgens both stimulate erythropoietin production by the kidneys and have a direct effect on the bone marrow stem cells. Clinically significant polycythemia is uncommon in hypogonadal men given androgen replacement except in patients who are likely to develop polycythemia, for example, those with chronic obstructive pulmonary disease or sleep apnea.

In hypogonadal men with other risk factors such as obesity and chronic obstructive airway disease sleep-related breathing disorders (sleep apnea) have been reported with androgen replacement. The usual doses of testosterone esters, when given to normal men, are not associated with changes in glucose or insulin levels. In recent years because of the action of testosterone on visceral fat, studies are in progress to study the effect of testosterone on decreasing insulin resistance in patients with the metabolic syndrome and diabetes type 2 *(40)*. It has been postulated, testosterone may reduce visceral fat and thus increase insulin sensitivity.

Benign prostatic hypotrophy and prostate cancer rarely occur in men who developed androgen deficiency before puberty. There is no clear evidence indicating that androgen replacement given to men who become hypogonadal after puberty increases the risk of prostatic disease. For all adult men, especially older men, on long-term androgen therapy, regular digital rectal examination and prostate-specific antigen levels should be monitored. If a suspicion of prostatic enlargement exists, a transrectal prostatic ultrasound should be performed and/or final needle biopsy of a suspicious nodule.

The effects of androgen on behavior and cognitive function have been topics of broad public interest. Anecdotal reports of androgen rage or increased aggressive behavior after androgen therapy have not been substantiated by controlled studies. A recent report *(61)* has shown improved mood, lessened depression, and general well being when hypogonadal men are treated with testosterone.

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