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

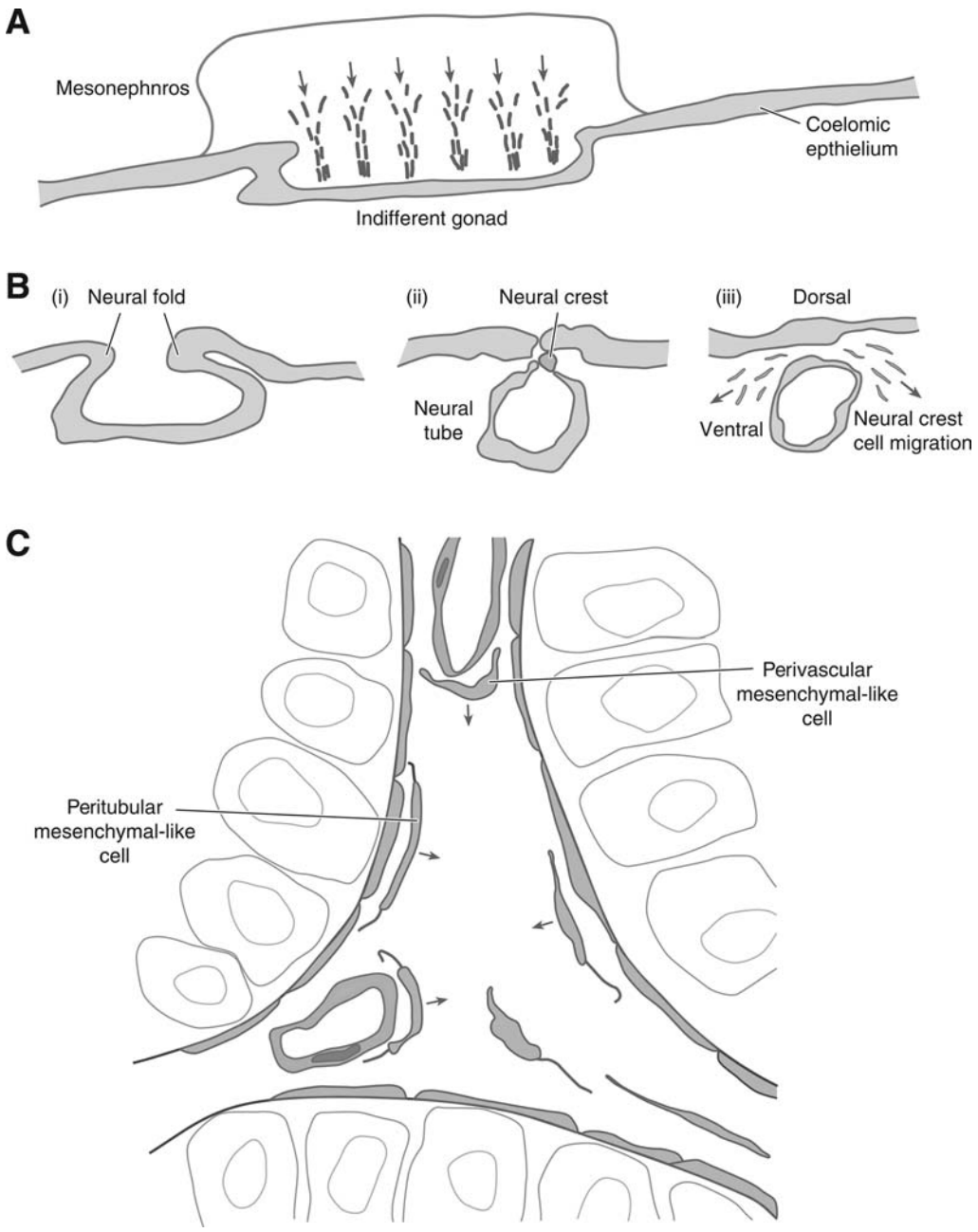

Fig. 1. Ontogeny of Leydig cells. In-migration of mesenchymal cells from the mesonephros is thought to furnish the Leydig stem cells to the interstitial spaces of the testes (arrows, **A**). However, this hypothesis has not been confirmed in studies of gonad-mesonephros host tissue recombinants, and 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

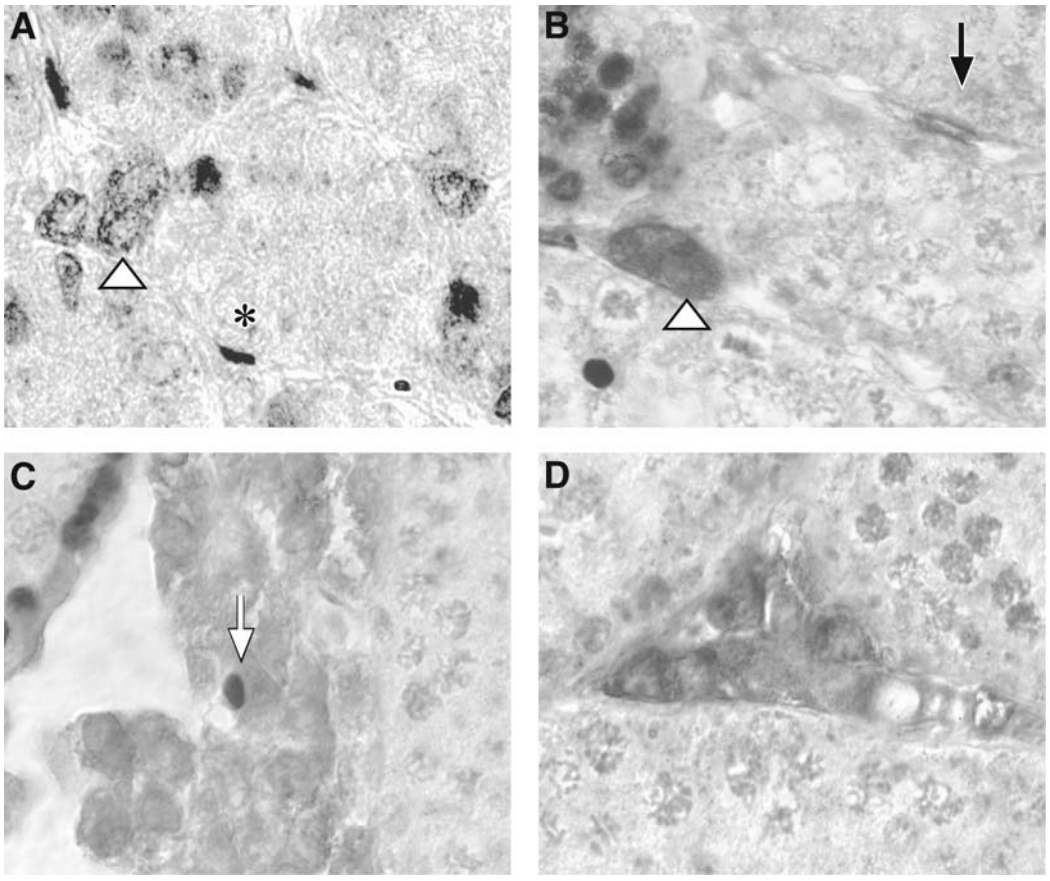

Fig. 2. Development of rat Leydig cells. Double immunolabeling of testicular cells for 3β-HSD and BrdU was assessed in sections of rat testes obtained on days 7 **(A)**, 14 **(B)**, 35 **(C)**, and 90 **(D)**. A cluster of 3β-HSD-positive presumptive fetal Leydig cells can be seen (brown staining, indicated 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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