Pergamon

0960-0760(95)00022-4

J. Steroid Biochem. Molec. Biol. Vol. 53, No. 1–6, pp. 61–68, 1995

0960-0760/95 $9.50 + 0.00

Differentiation of Adult Leydig Cells

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Adult Leydig cells originate within the testis postnatally. Their formation is a continuous process involving gradual transformation of progenitors into the mature cell type. Despite the gradual nature of these changes, studies of proliferation, differentiation and steroidogenic function in the rat Leydig cell led to the recognition of three distinct developmental stages in the adult Leydig cell lineage: Leydig cell progenitors, immature Leydig cells and adult Leydig cells. In the first stage, Leydig cell progenitors arise from active proliferation of mesenchymal-like stem cells in the testicular interstitium during the third week of postnatal life and are recognizable by the presence of Leydig cell markers such as histochemical staining for 3β-hydroxysteroid dehydrogenase (3β-HSD) and the present of luteinizing hormone (LH) receptors. They proliferate actively and by day 28 postpartum differentiate into immature Leydig cells. In the second stage, immature Leydig cells are morphologically recognizable as Leydig cells. They have an abundant smooth endoplasmic reticulum and are steroidogenically active, but primarily produce 5α-reduced androgens rather than testosterone. Immature Leydig cells divide only once, giving rise to the total adult Leydig cell population. In the third and final stage, adult Leydig cells are fully differentiated, primarily produce testosterone and rarely divide. LH and androgen act together to stimulate differentiation of Leydig cell progenitors into immature Leydig cells. Preliminary data indicate that insulin like growth factor-1 (IGF-1) acts subsequently in the transformation of immature Leydig cells into adult Leydig cells.

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INTRODUCTION

Two overlapping generations of Leydig cells populate the rat testis. The first generation develops *in utero* and its members are therefore called fetal Leydig cells. These cells produce the testosterone required for differentiation of the male urogenital system late in gestation. Fetal Leydig cells number about 200,000 per testis at their peak [1]. The second Leydig cell population predominates in the adult rat testis and its members are therefore called adult Leydig cells. Adult Leydig cells form during puberty and supply the testosterone required for the onset of spermatogenesis and maintenance of male reproductive function [2]. By the time of sexual maturity at day 56, the testis of the rat contains approx. 25 million adult Leydig cells. It is not known whether fetal Leydig cells persist in the adult testis. At their peak, however, they number less than 1% of the total number of adult Leydig cells, and do not contribute significantly to the hormonal output of the adult testis.

Proceedings of the IX International Congress on Hormonal Steroids, Dallas, Texas, U.S.A., 24–29 September 1994.
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Knowledge of the ontogeny of the adult Leydig cell and the development of its steroidogenic capacity has grown rapidly with the advent of methods to isolate and culture rat Leydig cells and their progenitors. This paper reviews our current understanding of the formation of adult Leydig cells in the rat. We discuss their embryonic origin, the commitment of stem cells to the Leydig cell lineage, and a three-staged model of Leydig cell differentiation. We present evidence that three key factors regulate Leydig cell differentiation: luteinizing hormone (LH), androgen, and insulin-like growth factor-1 (IGF-1).

EMBRYONIC ORIGIN OF LEYDIG CELLS

Both fetal [3] and adult Leydig cells [4, 5] descend from testicular mesenchymal-like stem cells, which suggests that their ontogenies are identical. Because fetal Leydig cells originate during embryogenesis, it is possible to trace their lineage back to the primordial germ layer (endoderm, mesoderm or ectoderm). Even so, conclusive data on the ultimate embryonic derivation of the Leydig cell are lacking. Two alternative sources have been proposed: mesoderm (either

mesonephrogenic mesenchyme or coelomic epithelium) and ectoderm (specifically the neural crest).

Most earlier studies of Leydig cell ontogeny reported that their stem cells originate in mesonephrogenic mesenchyme, a mesodermally derived tissue that in the male differentiates into the urogenital system. The fact that mesonephrogenic mesenchymal cells migrate into the gonad during embryogenesis and contribute to several somatic cell types [6], supports this theory. However in the chicken [7] and the mouse [6] the mesenchymal-like stem cells are derived from a source other than mesonephrogenic mesenchyme and Rodemer-Lenz has proposed that coelomic epithelium produces the Leydig stem cells [6]. Coelomic epithelium, also a mesodermally derived structure, forms the bulk of the gonadal ridge, and furnishes several types of somatic cells to the developing gonad prior to sexual differentiation [7].

In contrast to the above, the presence of neural cell adhesion molecule (NCAM) in adult Leydig cells suggests to some investigators that Leydig cells are descendants of neuro-ectoderm and specifically from neural crest cells that migrate into the gonodal ridge [8]. Although the presence of NCAM does not exclude the possibility of their mesonephrogenic origin, numerous other proteins normally found only in neuroectodermally derived tissues were immunocytochemically detected in adult mammalian Leydig cells [9, 10].

Currently there is no definitive assignment of Leydig cell ontogeny to mesodermal or neuro-ectodermal lineage. The mesenchymal-like morphology of Leydig stem cells is consistent with both neural-ectodermal and mesodermal origin [11]. This question may be resolved by tracking the neural crest lineage during embryonic development to determine whether it contributes to testicular somatic cells, specifically to Leydig stem cells.

POSTNATAL COMMITMENT OF STEM CELLS TO THE LEYDIG CELL LINEAGE

The early postnatal testicular interstitium contains a population of spindle-shaped undifferentiated cells designated "mesenchymal-like". From postnatal day 14 to 28 mesenchymal-like cells actively proliferate, while their numbers diminish, suggesting transformation to a new cell type. Leydig cells on the other hand increase in number without rapid proliferation, suggesting recruitment of another cell type to the Leydig cell population. Observations such as these led to the conclusion that stem cells of the adult Leydig cell population, are among the mesenchymal-like cells [4, 5]. They are most likely descendants of fetal Leydig stem cells that remain quiescent until stimulated to differentiate. By the second week of postnatal life, a cohort of mesenchymal-like stem cell progeny becomes committed to the Leydig cell lineage. The first signs of that commitment are evident on or about postnatal day 14 when some mesenchymal-like cells begin to express Leydig cell markers such as 3β-HSD [12, 13] activity and LH receptors [13].

A THREE STAGE MODEL OF LEYDIG CELL DIFFERENTIATION

The formation of the adult Leydig cell population from newly committed mesenchymal-like cells involves cell proliferation, morphological differentiation, and acquisition of testosterone producing capability. Analysis of this process was facilitated by the identification of three distinct stages of the differentiating Leydig cell: the Leydig cell progenitor, the immature Leydig cell, and the adult Leydig cell. Two consecutive transitions underlie the formation of adult Leydig cells. First, cell proliferation and an initial phase of differentiation produce the immature Leydig cells from Leydig cell progenitors. This transition, completed by day 28, accounts for half the total number of Leydig cells. Immature Leydig cells then undergo further differentiation and a single cell division. This second transition, completed by day 56, produces the full complement of 25 million adult Leydig cells. Figure 1 shows the time-course of postnatal Leydig cell differentiation and proliferation. The characteristics of a Leydig cell progenitor, immature Leydig cell and adult Leydig cell (described below) are typified at 21, 35, and 90 days, respectively. These forms are illustrated in Fig. 2, as they appear *in vivo*.

Leydig cell progenitors

As a direct result of the proliferation of mesenchymal-like stem cells in the testicular interstitium and their subsequent commitment to the Leydig cell lineage, Leydig cell progenitors are generated. They are recognizable as members of the Leydig cell lineage by their low level expression of Leydig cell markers including: histochemical staining for 3β-HSD, LH receptors and androgen production. Morphologically Leydig cell progenitors remain mesenchymal-like, with an elongated spindle-shape and little smooth ER.

Immature Leydig cells

By day 28 the Leydig cell progenitors transform from spindle-shaped to round and acquire numerous lipid inclusions, thus forming a population of immature Leydig cells. During this process, their smooth ER expands greatly, conferring the typical ultrastructural morphology of a Leydig cell. As a result the number of morphologically recognizable Leydig cells per testis increases from 0.4 to 13.4 million [4]. Concurrent with the expansion of smooth ER, the cells develop a capacity for steroidogenesis [14]. The levels of 3β-HSD [12, 15, 16], cholesterol-side-chain-cleavage

enzyme [17], and 17α-hydroxylase [17] increase in tandem with the expansion of the smooth ER. Acquisition of testosterone producing ability, however, not only entails an increase in testosterone biosynthetic activity, but also involves a decrease in activities of testosterone metabolizing enzymes [18, 19]. Immature Leydig cells primarily produce the androgen metabolite, 5α-androstane-3α, 17β-diol (ADIOL), because they possess high levels of androgen-metabolizing enzyme activity. Consistent with the trends in enzyme activity, mRNA levels for 3α-hydroxysteroid dehydrogenase (3α-HSD) [17] and 5α-reductase [16], enzymes that metabolize precursors of testosterone to ADIOL, are higher in the immature Leydig cell than the adult Leydig cell.

Adult Leydig cells

The Leydig cell population doubles only once from day 28 to day 56, producing the total adult population of approx. 25 million Leydig cells per testis. The activities of androgen metabolizing enzymes decline by day 56 [18, 19], as immature Leydig cells differentiate into adult Leydig cells. The decrease in androgen metabolism and continued increase in levels of testosterone biosynthetic enzymes culminate in the predominance of testosterone over ADIOL production by adult Leydig cells. In a 90 day adult Leydig cell, testosterone production is 150 times greater than in a Leydig cell progenitor at 21 days of age, and 5 times greater than in an immature Leydig cell at 35 days of age. Compared to the immature stage, the adult Leydig cell has a higher abundance of smooth ER, is largely devoid of lipid inclusions [14] and has a more prominent nucleolus.

Adult Leydig cells do not normally proliferate [20], but can be regenerated if destroyed. The adult population of Leydig cells is completely regenerated within 7 weeks of its destruction by ethanedimethane sulfonate

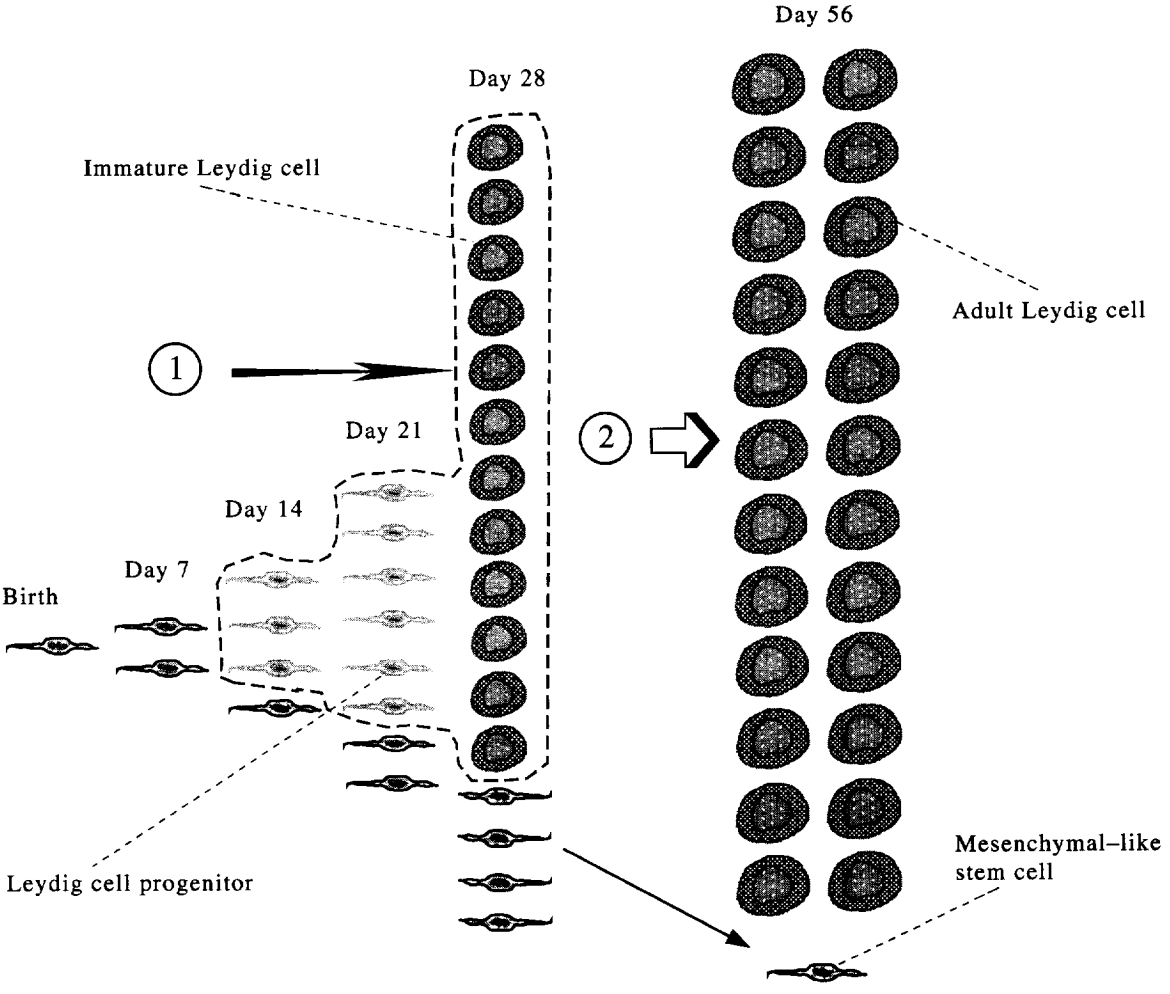
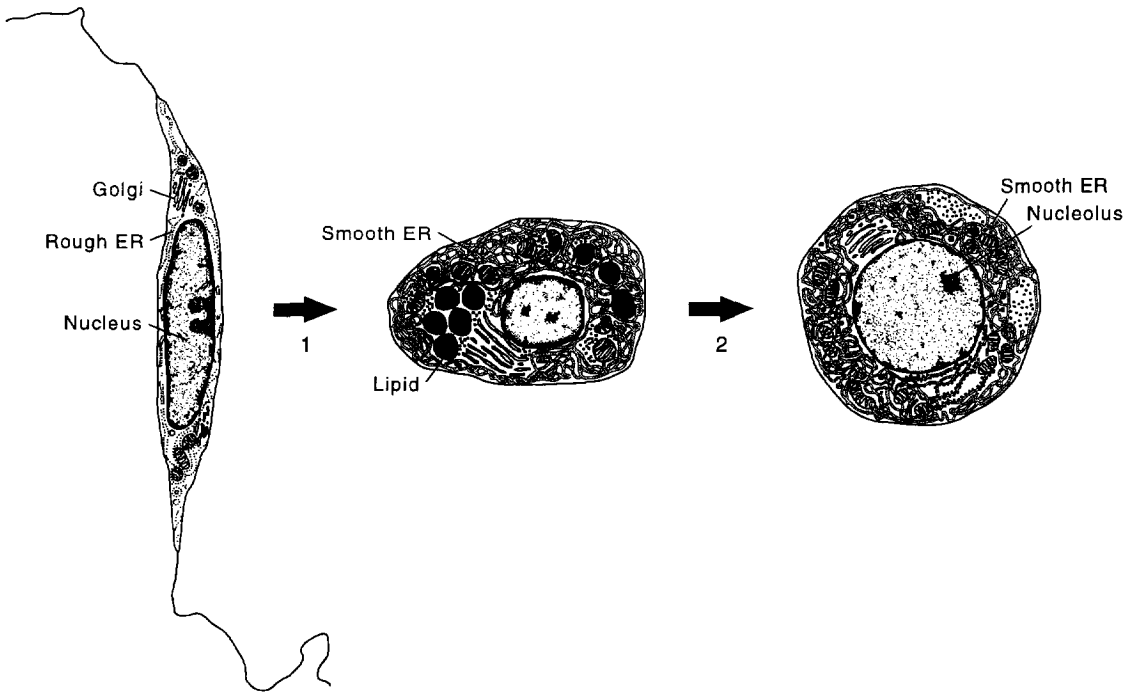


Fig. 1. The roles of proliferation and differentiation in Leydig cell development. The spindle-shaped mesenchymal-like cells divide actively, doubling approximately every 7 days. By day 14 a subset of these cells become progenitors committed to the Leydig cell lineage (surrounded by the dashed line). Between days 14 and 28 about half of the adult number of Leydig cells is generated as the progenitor cells, in the first of two phases of differentiation, transform into immature Leydig cells (arrow #1). Between days 28 and 56, the immature Leydig cells divide once, in the second phase of differentiation and transform into adult Leydig cells (arrow #2). A residual number of mesenchymal-like stem cells persist in the adult testis (unlabeled arrow).



CELLULAR ATTRIBUTES:	LEYDIG CELL PROGENITOR	IMMATURE LEYDIG CELL	ADULT LEYDIG CELL
Mitotic activity	High	Divides once	Probably none
Morphology	Spindle-shaped	Round with lipid droplets	Round without lipid droplets
Smooth ER	Low	High	Highest
Testosterone Metabolism	High	Highest	Low
Testosterone Synthesis	Low	Intermediate	High
Age when cell is typical	21 days	35 days	90 days
LH Receptor Number	Low	Intermediate	High
Androgen Receptor Number	High	High	Low

Fig. 2. Differentiation of the adult Leydig cell in the rat. A progenitor cell, immature Leydig cell, and adult Leydig cell are depicted as they would appear *in situ* on days 21, 35, 90, respectively. The characteristics of each cell type as described in text make it readily distinguishable from the other two.

(EDS), an agent specifically lethal to adult Leydig cells [21]. Progenitor cells and immature Leydig cells, are not commonly observed in the testis immediately following EDS administration. Therefore regeneration probably results from commitment of Leydig stem cells, that persist in the testis throughout adult life.

HORMONAL CONTROL OF LEYDIG CELL DIFFERENTIATION

A multiplicity of potential regulators of the Leydig cell are present in the testis (surveyed by Saez [22]), some of which influence the immature Leydig cell when tested *in vitro* [22]. The focus of the present

review is on three key regulators from this list: androgen, and IGF-1. LH is the chief regulator of adult Leydig cells and is also involved in their differentiation. Other factors are required, however, including androgen, acting in an autocrine fashion, and IGF-1, which attains its highest testicular concentration during puberty [23]. LH, androgen and IGF-1 not only stimulate differentiated function in the Leydig cell, but the absence of each at critical times during puberty interferes with normal Leydig cell development.

Luteinizing hormone

LH stimulates testosterone production by inducing transcription of biosynthetic enzymes [24] and maintains adult Leydig cell morphology [25–28]. A growing body of evidence shows that LH also regulates Leydig cell development, but whether it does so by promoting differentiation or proliferation or both was unclear until recently. Studies reviewed below indicate that LH has a critical role in differentiation, but not proliferation.

In the immature hypophysectomized rat, exogenous LH replacement increases the number of morphologically recognizable Leydig cells, however, not to the levels seen in intact control rats [29]. Thus LH, while important, is not the sole regulator of Leydig cell differentiation. FSH replacement increases the number of Leydig cells to a lesser degree, but is more effective than LH in stimulating testosterone production, responsiveness to LH, and 3β-HSD activity [30]. Sertoli cells are the main site of FSH binding in the testis [31]; therefore the effect of FSH on Leydig cell differentiation is probably mediated indirectly via Sertoli cell factors. Leydig cell progenitors possess very few LH receptors (1/16 the number compared to adult Leydig cells) [13] and are therefore relatively insensitive to LH. LH is therefore probably less important during early Leydig differentiation, and acquires more influence as sensitivity of the Leydig cell to LH increases.

Khan *et al.* have compared LH with three growth factors, IGF-1, transforming growth factor-α and transforming growth factor-β for stimulation of radiolabelled thymidine incorporation (as a measure of cell proliferation) by Leydig cells isolated from 21-day-old rats. LH induced slightly more thymidine uptake than control, but the effects of the three growth factors were more dramatic [32]. Much of the evidence that proliferation of mesenchymal-like stem cells is governed by LH is based circumstantially on Leydig cell repopulation after treatment of the adult testis with EDS. After EDS-induced destruction of Leydig cells, LH levels rise due to the temporary loss of feedback suppression of the hypothalamic–pituitary axis by androgen. During this period of high LH, mesenchymal-like stem cells rapidly divide [33]. However, mesenchymal-like stem cells still proliferate in hypophysectomized animals after EDS treatment, though they do not develop 3β-HSD enzyme activity until exogenous LH is added [33]. This indicates that LH is not required for early proliferative activity of Leydig stem cells, but is required for subsequent differentiation of these cells. Further evidence that proliferation of Leydig stem cells is not dependent on LH comes from studies of transitory hypothyroidism induced by treatment with the goitrogen, 6-propyl-2-thiouracil (PTU). Neonatal exposure to PTU permanently suppresses serum LH [34], but increases Leydig cell numbers [35, 36].

It is possible than androgen acts as a negative regulator of Leydig stem cell division and that low levels of androgen, rather than high levels of LH are responsible for proliferation of Leydig stem cells. The decline in androgen levels that occurs in EDS-treated animals might signal proliferation of mesenchymal-like stem cells. Proliferation is then prevented by a testosterone-releasing implant and not by hypophysectomy because the former raises serum androgen levels while the latter does not. Similarly, neonatally hypothyroid rats have low LH levels and therefore produce less androgen per Leydig cell. We speculate that this produces a higher drive for Leydig stem cell proliferation. Despite an increase in Leydig cell number, normal serum testosterone levels are maintained in these rats [35], suggesting that once normal testosterone levels are reached the drive to increase Leydig cell numbers ceases. Regulating Leydig cell proliferation may be an important physiological mechanism of attaining normal serum testosterone and signalling the end of the proliferative phase of mesenchymal-like cells by postnatal day 28. The hypothesis that early proliferation of stem cells is regulated inversely by levels of androgen instead of directly by LH levels is consistent with the fact that while there is no distinct pubertal LH peak in rats [37], the slowing of Leydig cell proliferation coincides with development of androgen producing capacity by Leydig cells.

The role of LH during development of Leydig cells can be summarized as follows: LH is essential in Leydig cell differentiation and maintenance of the differentiated state of the Leydig cell. Other factors are also important in differentiation of Leydig cells, especially in inducing Leydig cell sensitivity to LH. LH has only a marginal effect on Leydig cell proliferation. We speculate, however, that androgen is an important negative regulator of Leydig stem cell proliferation. An association between inhibition of Leydig cell proliferation by androgen, and induction of their differentiation (see next section), is consistent with the coupling of growth arrest and differentiation which is known to occur in mesenchymal stem cells [38].

Androgen

Several models have been employed to investigate androgen effects on Leydig cell differentiation. They have established the importance of autocrine regulation by androgen in the differentiation of Leydig cell progenitors into immature Leydig cells.

Isolated Leydig cell progenitors increased their capacity for testosterone production after 3 days *in vitro* when treated with the combination of LH and dihydrotestosterone (DHT). Either factor alone did not significantly increase testosterone production [39]. This potent stimulation of testosterone production in Leydig cell progenitors by androgen plus LH contrasts with androgen's inhibitory effect on steroid production in adult Leydig cells [40–43]. We have recently shown that androgen stimulates expression of LH receptors. Both androgen production and expression of LH receptors are markers of a differentiated Leydig cell. Further evidence of the role of androgen in Leydig cell differentiation has been observed in experimental models that induce androgen deprivation during pubertal development: androgen receptor antagonism, GnRH antagonism, and androgen insensitivity.

The anti-androgen, cyproterone acetate produces a state of androgen deprivation by blocking the androgen receptor. When administered *in vivo*, this agent delays the appearance of 3β-HSD staining in Leydig cell progenitors [44]. However, as cyproterone acetate binds to many other steroid receptors [45], it is possible that its effect on Leydig cell development is not due to blockade of the androgen receptor.

Treatment with GnRH antagonist suppresses LH and thereby induces a state of androgen deprivation. GnRH antagonism for 3 weeks *in vivo* starting at 21 days causes morphological degeneration of Leydig cells, consisting of decreased nuclear and cytoplasmic volume and distortion of nuclear shape. Testosterone replacement alone completely restores Leydig cell morphology to normal, indicating that LH induces differentiated Leydig cell morphology in part by stimulating androgen production [28].

In the male *Tfm* mouse, which is insensitive to androgen because of a mutation that renders androgen receptors nonfunctional, low testosterone (12% of normal [46]) is associated with deficient 17-ketosteroid reductase and 17α-hydroxylase enzyme activities [47]. In addition, Leydig cells of the *Tfm* rat have fewer than 20% of the normal number of LH receptor, produce less testosterone, and are unresponsive to hCG [48]. These deficiences in the *Tfm* animal probably result from lack of androgen stimulation of Leydig cell progenitors and not high LH levels which would be expected to increase testosterone production [46].

Serum LH levels are normal in the first of these three androgen-deficiency models, depressed in the second and elevated in the third. All of the methods result in abnormalities of Leydig cell development. Therefore, regardless of LH levels, androgen deprivation is correlated with Leydig cell abnormalities. This strongly suggests that androgen is involved in Leydig cell differentiation.

The pattern of androgen receptor expression reflects the importance of androgen action on Leydig cell differentiation. In the Leydig cells, levels of androgen receptor mRNA and protein peak in the immature animal, and decline with sexual maturity [17]. In the testicular interstitium both DHT and testosterone are effective agonists at the androgen receptor [49]. Studies of Leydig cell progenitors *in vitro* show that, when the conversion of testosterone to DHT is suppressed by a 5α-reductase inhibitor, testosterone production is not affected [50]. This suggests that testosterone and DHT exhibit a similar stimulation of testosterone production.

The above experiments indicate that Leydig cell differentiation is stimulated synergistically by LH and androgen. LH stimulates testosterone production, which in turn stimulates further testosterone production (positive feedback) and differentiation of the Leydig cell. Androgen facilitation of testosterone production by Leydig cell progenitors does not occur in the presence of weak androgens such as 5α-androstane-3α,17β-diol (ADIOL) [39]. The ability of LH plus DHT to stimulate testosterone production in the Leydig cell progenitor increases when metabolism by 3α-HSD is blocked with 1-(4'-nitrophenyl)-2-propen-1-ol, an inactivator of 3α-HSD [40]. This indicates that weak androgens do not stimulate androgen production of the progenitor Leydig cell. Consequently, 3α-HSD activity in the immature Leydig cell results in lower stimulation of testosterone production. By reducing the positive feedback of androgen on androgen production, 3α-HSD in immature animals may delay a rise in serum testosterone levels until it is developmentally appropriate for androgenization to occur.

In summary, androgen plus LH stimulates testosterone production, whereas androgen deprivation results in deficient Leydig cell development. LH facilitates autocrine action of androgen on Leydig cell progenitors, which results in their differentiation into immature Leydig cells. Recent evidence that androgen potentiates the expression of LH receptors in Leydig cell progenitors [51] indicates that androgen and LH are mutually inductive, with LH stimulating androgen production, which in turn increases the sensitivity of the cell to LH.

IGF-1 (Insulin-like growth factor-1)

IGF-1, as the mediator of growth hormone action, is essential for normal mammalian growth and development [52]. In addition to mediating the action of growth hormone, IGF-1 acts independently as a paracrine and autocrine factor [53]. The possibility that IGF-1 promotes differentiation of the Leydig cell is suggested by the fact that immature Leydig cells possess high affinity IGF-1 receptors [54], and testicular levels of IGF-1 peak during puberty [55]. Testicular IGF-1 mRNA levels increase in immature rats after treatment with exogenous LH, FSH and GH *in vivo* [55]. This is consistent with autocrine secretion by Leydig cells, paracrine secretion by Sertoli cells and GH-stimulated hormonal secretion from the liver as significant sources of testicular IGF-1. In addition,

studies have shown that both Sertoli cells [55, 56] and Leydig cells [55] secrete IGF-1 *in vitro*. Thus IGF-1 can act on Leydig cells during puberty as a hormonal, autocrine and paracrine factor.

Two lines of evidence suggest that IGF-1 regulates the transformation of immature Leydig cells into adult Leydig cells. First, IGF-1, in the presence of LH, stimulates testosterone production more effectively in immature Leydig cells than in adult Leydig cells [57]. The dependence of this effect on LH may be due to an LH-induced increase in IGF-1 receptor number [54]. Second, Leydig cells of mice with a targeted deletion of the IGF-1 gene remain functionally and structurally immature. In the absence of IGF-1, the Leydig cell contains less smooth ER and produces lower levels of testosterone, but higher levels of androstenedione [Hardy, unpublished observations]. High androstenedione production is characteristic of an immature mouse Leydig cell [58], suggesting that IGF-1 is necessary for the transition from the immature to the mature pattern of steroidogenesis. Increasing expression of 17-ketosteroid reductase, the enzyme that catalyzes the conversion of androstenedione to testosterone, is involved in this transition and therefore a likely target of IGF-1 action.

CONCLUSION

After the commitment of mesenchymal-like stem cells to the Leydig cell lineage, there is a progression through three distinct stages of development: Leydig cell progenitor, immature Leydig cell, and adult Leydig cell. LH and androgen are key regulators of the transition from Leydig cell progenitor to immature Leydig cell. Androgen may also be involved in cessation of proliferation that occurs during this transition. Evidence suggests that IGF-1 influences the final transition from immature to adult Leydig cell.

Complete understanding of Leydig cell differentiation is not yet at hand, and the factors that regulate the earliest stage of differentiation, the commitment of mesenchymal-like stem cells, remain elusive. For example, LH, androgen and IGF-1, though involved at later stages, cannot be the factors that regulate the commitment of stem cells to the Leydig cell lineage. LH is essential for complete differentiation, but the initial proliferation of mesenchymal-like stem cells can occur when LH is absent, and animals with low LH still develop Leydig cells. Similarly, animals with androgen insensitivity or deletion of the IGF-1 gene develop Leydig cells. Despite the incomplete status of our present understanding, the three stage model of differentiation provides a useful framework for future studies of the hormonal regulation of the Leydig cell.

Acknowledgements—We thank Chantal Manon Sottas for skilled technical assistance. This work was supported in part by The Population Council and the NIH (R29HD-32588).

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