REVIEW

Origin, proliferation and differentiation of Leydig cells

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INTRODUCTION

Mammalian sex determination involves complex interacting networks of cellular and hormonal signals leading to the development of male or female phenotypes. Three main sequential processes are involved: first, the establishment of chromosomal sex at fecundation (genetic sex); second, the development of the undifferentiated gonad into either testes or ovaries (gonadal sex); and third, differentiation of male or female internal and external genitalia (phenotypic sex). Male phenotype is controlled by two testicular hormones, the anti-Müllerian hormone (AMH) secreted by fetal Sertoli cells which induces regression of the Müllerian ducts, and testosterone produced by Leydig cells which induces differentiation of the Wolffian ducts into male reproductive organs, although conversion of testosterone into dehydrotestosterone is required for masculinization of the external genitalia. In the absence of testes, and therefore in the absence of both AMH and testosterone, the Wolffian ducts regress, creating a permissive environment for the differentiation of the Müllerian ducts and, thereby, female reproductive organs.

MOLECULAR DETERMINATION OF MAMMALIAN SEX

In mammals, the choice between male and female development is controlled by the sex chromosomes; the presence of a Y chromosome results in male development, regardless of the number of X chromosomes (Ford *et al*. 1959, Jacobs & Strong 1959). Experiments performed by Alfred Jost and colleagues in the 1940s demonstrating that castration of rabbit embryos of both chromosomal sexes induced female development, indicate that the presence of the testis is necessary for the development of male characteristics (Jost 1947, Jost *et al*. 1973). It was postulated that there must be a dominant gene or genes on the Y chromosome required for testis development. This genetic entity was named the testis determining factor (TDF). In male meiosis, the X and Y chromosomes pair at the tip of their short arms in a region known as the pseudoautosomal region, and this pairing is essential for correct segregation of the X and Y chromosomes (Ellis 1991). In every male meiotic event, a single recombination occurs in the pseudoautosomal region, and this event maintains homology between the X and Y shared region. Occasionally, recombination extends beyond the pseudoautosomal region, so that Y-specific sequences are transferred to the X chromosome and X-specific sequences are transferred to the Y chromosome. Such aberrant recombinations produce XX males, who possess Y-specific sequences, and XY females, who have lost TDF. The size of Y DNA found in XX males varies but can be as small as 35–40 kb (Palmer *et al*. 1989, Sinclair *et al*. 1990). Of the many probes prepared from this region only one recognized a Y-specific sequence conserved among all eutherian DNA samples. This probe derived from a fragment located 5 kb proximal to the pseudoautosomal boundary. When this region was cloned and sequenced only one candidate sequence was identified, named SRY (sex-determining region of Y gene), which contains a 669 bp open reading frame (Sinclair *et al*. 1990). The evidence equating SRY with TDF was afforded by three types of studies: (1) demonstration that many XY females had point mutations or small deletions of SRY (reviewed in Goodfellow &

Journal of Molecular Endocrinology (1998) **20,** 1–25 *Printed in Great Britain*
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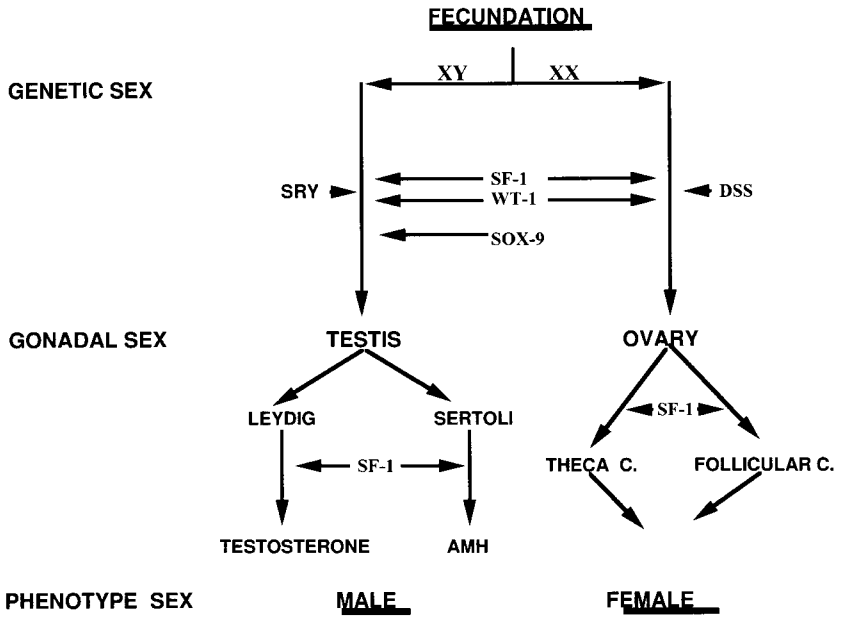


FIGURE 1. Schematic representation of steps in mammalian sex determination and the genes implicated in each step. SRY (sex-determining region Y gene); SF-1 (steroidogenic factor-1); SOX-9 (SRY-related HMG-box containing gene 9); WT-1 (Wilms' tumor); DSS (dosage sensitive sex reversal); AMH (anti-Müllerian hormone).

Lovell-Badge 1993); (2) production of XX male mice in transgenic animals expressing SRY (Koopman *et al*. 1991); and (3) expression of SRY transcripts only in somatic cells of the embryonic male gonadal ridge (Hacker *et al*. 1995).

Although biochemical studies have failed to identify genes that regulate SRY and genes directly regulated by SRY, at least three gene products appear to play a crucial role in the development of the undifferentiated gonad (Fig. 1), namely the orphan nuclear receptor gene SF-1 (steroidogenic factor-1) (Ikeda *et al*. 1993), Wilms' tumor suppressor gene (WT1) (Hastie 1994) and a high mobility group (HMG) family member, which is closely related to SRY and known as SOX9 (Foster *et al*. 1994, Wagner *et al*. 1994).

In mice, gonadal expression of SF-1 begins from 9 days post-coitum (dpc) and is extinguished by 12·5 dpc in females, but in males expression persists (Ikeda *et al*. 1994). The expression profile of SF-1 in gonads is parallel to that of AMH, and SF-1 is probably involved in the regulation of AMH expression (Shen *et al*. 1994). Moreover, SF-1 knockout mice lack both gonads and adrenal glands (both the gonadal ridge and the adrenal primordium arrest in development and degenerate) (Luo *et al*. 1994).

The WT1 gene was originally isolated as an oncogene involved in the childhood kidney cancer, i.e. Wilms' tumor (Call *et al*. 1990, Rose *et al*. 1990). In addition to being expressed in the developing renal tract, its transcripts are also found in the mouse gonadal ridge from 9 dpc in both males and females (Pelletier *et al*. 1991*b*). Further evidence for a role in gonadal formation derives from the discovery of a deletion in chromosome 11 in patients with WAGR syndrome (Wilms' tumor, aniridia, genito-urinary abnormalities and mental retardation) (Haber & Housman 1992) and heterozygous mutations of WT1 in patients with Denys–Drash syndrome (Wilms' tumors, glomerular nephropathy and varying degrees of abnormal gonadal and genital development) (Pelletier *et al*. 1991*a*). Moreover, WT1 knockout mice die during the embryonic period, following failure of kidney organogenesis and gonadal degeneration (Kreidberg *et al*. 1993).

The third gene product implicated in testicular development is SOX9. Mutations in the SOX9 gene, which is located in the long arm of human chromosome 17, have been linked to a severe dwarfism syndrome known as campomelic dysplasia (Foster *et al*. 1994, Wagner *et al*. 1994). Patients with this syndrome display a number of congenital skeletal abnormalities and more than 75% of XY patients show sex reversal with a gradation of genital defects. In all patients studied so far, the

patients with or without sex reversal are heterozygous for the mutations. Recent studies have shown that SOX9 is expressed in the genital ridge of both XY and XX embryos at about 10·5 dpc. However, after 11·5 dpc, its expression is very abundant in genital ridges from XY embryos, but is absent from those of XX embryos (Morais da Silva *et al*. 1996).

Evidence that a fourth gene located in the X chromosome might be involved in gonadal differentiation comes from the finding that duplication of Xp in association with a normal Y chromosome containing an intact SRY, can result in male to female sex reversal (Bardoni *et al*. 1993, Am *et al*. 1994). This dosage-sensitive sex reversal locus (DSS) was mapped to a region on Xp21 adjacent to the adrenal hypoplasia congenital locus (AHC) (Bardoni *et al*. 1994). Although DSS can interfere with testis determination when duplicated, it is not essential for testis formation as 46XY individuals carrying deletions of Xp21 region have a male phenotype. Therefore, it was proposed that DSS could be required for ovarian development, by either promoting differentiation of ovarian cell types, repressing differentiation of testicular cells, or both (Bardoni *et al*. 1994). A candidate gene isolated from DSS region, DAX1 (DSS–AHC critical region on the X, gene 1) (Muscatelli *et al*. 1994, Zanaria *et al*. 1994), was found to encode an orphan nuclear hormone receptor. Deletion or point mutations of DAX1 are responsible for AHC. DAX1 is expressed in adrenal primordium, developing hypothalamus and genital ridge of both male and female (11·5 dpc). Moreover, after 12 dpc, DAX1 expression in the male gonad decreases dramatically as testis cords begin to appear, but persists in the developing ovary (Swain *et al*. 1996). This pattern of expression is consistent with DAX1 being equivalent to DSS.

ORIGIN AND DEVELOPMENT OF FETAL LEYDIG CELLS

In mammals, the ontogenesis of Leydig cell function involves at least two generations of cells. The first generation develops during fetal life and these fetal Leydig cells are responsible for the masculinization of the male urogenital system. These cells regress thereafter, although in the rat some of these cells may persist in adult life. The second Leydig cell populations appear during puberty and produce the testosterone required for the onset of spermatogenesis and maintenance of male reproductive function.

Rat

The first fetal Leydig cells differentiate relatively late in the course of testis formation. In the rat, seminiferous cords begin to form at 13 days 9 h (dpc) by the emergence of a new type of large 'clear' cells (Sertoli cell precursors), which aggregate and surround the germ cells in the forming seminiferous cord (Magre & Jost 1980, 1984, Jost *et al*. 1981). Thereafter, the Leydig cells appear in the interstitial region by differentiation from mesenchymal-like stem cells (Byskow 1986). However, conclusive data on the ultimate embryonic origin of Leydig stem cells are lacking (Benton *et al*. 1995). Early studies proposed that Leydig stem cells are mesodermal, appearing first in the mesonephros, and then migrating into the presumptive interstitial tissue (Wartenberg 1978). A more recent hypothesis is that Leydig stem cells derive from neural crest. This hypothesis is based on evidence that Leydig cells express several neural specific proteins such as neural cell adhesion molecule, neurofilament protein 200, and microtubular-associated protein (Mayerhofer *et al*. 1992*a*, 1996, Davidoff *et al*. 1993, Middendorff *et al*. 1993). In the rat morphological (Jost *et al*. 1981) and functional differentiation, the ability to produce testosterone (Warren *et al*. 1973; Habert & Picon 1984) of Leydig cells is first observable on day 15·5 dpc (Fig. 2).

The signal(s) triggering the initial differentiation of rat fetal Leydig cells is unknown. Although, as indicated above, the Y chromosome containing an intact SRY is absolutely required to induce the differentiation of the gonadal ridge into testis, a sexual genetic control of Leydig cell differentiation is unlikely since in XX/XY chimeric mouse testes XX cells contribute to the formation of Leydig cells (Burgoyne *et al*. 1988). The initial differentiation of fetal rat Leydig cells is also gonadotropin independent, since pituitary luteinizing hormone (LH) cannot be detected until day 16·5 (Aubert *et al*. 1985) (Table 1) and gonadal anlage removed at 12 days 16 h or 13 days 9 h (dpc), and cultured in hormone-free medium, complete their morphological and endocrine differentiation within 3 days (Agelopoulou *et al*. 1984, Gangnerau & Picon 1987, Jost *et al*. 1988). Furthermore, although early studies described the presence of biological and immunological chorionic gonadotropin (CG)-like activity in the rat placenta (Blank & Dufau 1983), subsequent studies failed to detect such activity (Wurzel *et al*. 1983, Habert & Picon 1990). Indeed, with the exception of the primates and equine, there is no CG gene in other mammalian species.

After the initial differentiation of fetal Leydig cells, testicular steroidogenesis increases markedly

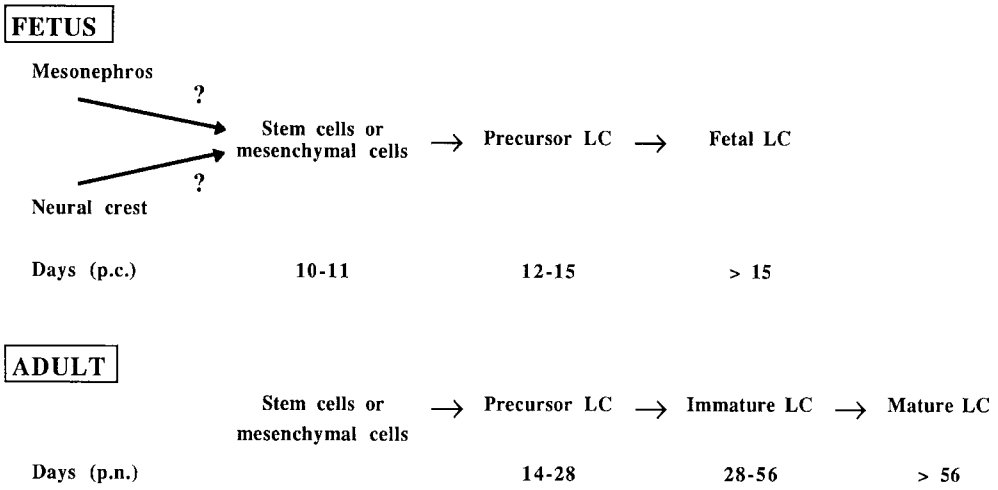


FIGURE 2. Origin and differentiation of Leydig cells (LC) in the rat. p.c., post-coitum; p.n., post-natal.

TABLE 1. Developmental maturation of the rat fetal pituitary–testicular axis

Fetal age (days)	Functions	Number of Leydig cells	LH dependence
13·5	Conversion of progesterone and dehydroepiandrosterone→testosterone	—	Independent
15·5	Testosterone synthesis LHR and LH responses	—	
16	Onset of LH synthesis	$0{\cdot}25 \times 10^5$	
18	Maximal testosterone production	$0{\cdot}60 \times 10^5$	
19	LH detectable in plasma Onset of testosterone decline	—	Dependent
21	Maximum LH in plasma	$1{\cdot}20 \times 10^5$	

at the same time as the number of Leydig cells increases from $0{\cdot}25 \times 10^5$ cells per testis on day 16·5 to $1{\cdot}2 \times 10^5$ cells per testis on day 21 (Kerr & Knell 1988). This increase is due mainly to differentiation of stem cells, since fetal Leydig cells have no mitotic activity (Orth 1982). The results observed after decapitation of rat fetuses at several days of gestation, indicate that the development and function of fetal Leydig cells is gonadotropin independent until day 18 and comes under control of fetal LH thereafter (Eguchi *et al*. 1978, Habert & Picon 1982) (Table 1). Although there is some indication that Sertoli cell-secreted factors (Byskow 1986) and the extracellular matrix (Jost *et al*. 1988) might be involved in the initial, gonadotropin-independent, development of fetal Leydig cells, the nature of such factors is unknown. Interestingly, despite the fact that plasma LH levels remain high at the end of gestation, and the number of Leydig cells increases, both *in vivo* and *in vitro* studies have demonstrated a functional regression of Leydig cells beginning during late fetal life and remaining after birth (Habert & Picon 1982, Tapanainen *et al*. 1984, Habert & Brignaschi 1991, Habert *et al*. 1992, Huhtaniemi & Pelliniemi 1992, Habert 1993). The factors responsible for this regression are unknown but transforming growth factor beta (TGFβ) might be involved since its expression by fetal Leydig cells appears on day 16·5, greatly increases during late fetal life and persists until postnatal day 20 (Gautier *et al*. 1994). Moreover, this factor is a strong inhibitor of Leydig cell functions (see below).

Human

The differentiation of the human male gonads begins in the sixth week of gestation with the gradual development of gonadal blastema into

TABLE 2. Origin and differentiation of Leydig cells (LC) in human: stem cells→precursor LC/mesenchymal cells/infantile LC→immature LC/pubertal LC→mature LC

	Age	Number of LC
Fetal		
Proliferation and differentiation	8–14 weeks	—
Mature	14–18 weeks	48×10^6
Stationary	18–24 weeks	48×10^6
Involution	24–38 weeks	18×10^6
Postnatal		
Neonatal	0–1 years	—
Infantile	1–8 years	—
Pubertal	8–15 years	—
Adult	>15 years	5×10^8

TABLE 3. Developmental maturation of the human fetal pituitary–testicular axis

	Functions	Hormone dependence
Fetal age (weeks)		
2	hCG secretion	
7–8	Onset of LC differentiation Onset of testosterone synthesis	LH/hCG independent
10	Onset of LH synthesis LHR in testis	hCG dependent
11	LH in plasma	
12–15	Maximum hCG in plasma Maximum testosterone synthesis	
22–24	Maximum LH in plasma	LH dependent
24–38	↓ LH and hCG in plasma ↓ LC number ↓ Testosterone in plasma	

testicular cords and the interstitium (Gondos 1980). The fetal Leydig precursors become identifiable during the eighth week of gestation (Table 2). Cytodifferentiation begins with an increase in cytoplasmic volume, development of smooth endoplasmic reticulum, an increase in the number and the size of the mitochondria, enlargement of the nucleus, and accumulation of lipid droplets. Cytodifferentiating Leydig cells can be observed among fully differentiated ones until the 10th week of fetal life (Rabinovici & Jaffe 1990, Huhtaniemi & Pelliniemi 1992). The functional differentiation of Leydig cells seems to begin before the first signs of cytodifferentiation, since testosterone is detected in the fetal human testis at 6 to 7 weeks of gestation (Tapanainen *et al*. 1981). The ontogenesis of the pituitary–testicular axis in humans is summarized in Table 3. It is clear that in the human, as in all other mammals studied, LH does not control initial Leydig cell differentiation since the onset of steroid production by the fetal testis precedes that of LH secretion by the hypophysis (Reyes *et al*. 1989). However, in humans, because human CG (hCG) is produced by the placenta well before testicular development, it was thought that this hormone might be responsible for the initial development of Leydig cells. Against this hypothesis is the fact that one patient with an inactivating mutation of LH/hCG receptor producing complete loss of function had some development of vas deferens and epididymis associated with female external genitalia (Kremer *et al*. 1995). Since development of the Wolffian ducts absolutely requires the presence of testosterone, the above finding indicates that in this patient the initial functional differentiation of Leydig cells and therefore the secretion of

testosterone were hCG independent. Moreover, they also indicate that after this initial phase, hCG is required for Leydig cells to produce enough testosterone for masculinization of the external genitalia. Indeed, *in vitro* studies have shown that hCG is able to stimulate testosterone production by human and primate fetal Leydig cells (Rabinovici & Jaffe 1990). During late fetal life, as in the rat, testicular steroidogenic activity appears to be under pituitary control since anencephalic human fetuses have a reduced number of Leydig cells and a subnormal testicular steroidogenesis (Rabinovici & Jaffe 1990) and since gonadotropin insufficiency is very often associated with micropenis (Migeon *et al*. 1994). After birth, in both human (Forest *et al*. 1973) and non-human primates (Fouquet *et al*. 1984), there is a second testosterone surge and it is associated with the development of a second wave of Leydig cells (Fouquet *et al*. 1984, Codesal *et al*. 1990, Prince 1990). Thereafter, the number of Leydig cells decreases, so that very few Leydig cells remain by the end of the first year of life (Codesal *et al*. 1990, Prince 1990). Including the pubertal wave, there are thus three waves of Leydig cell development in man. In the pig, Leydig cell development also occurs in three waves (Van Straaten & Wensing 1978).

ORIGIN AND DIFFERENTIATION OF ADULT LEYDIG CELLS

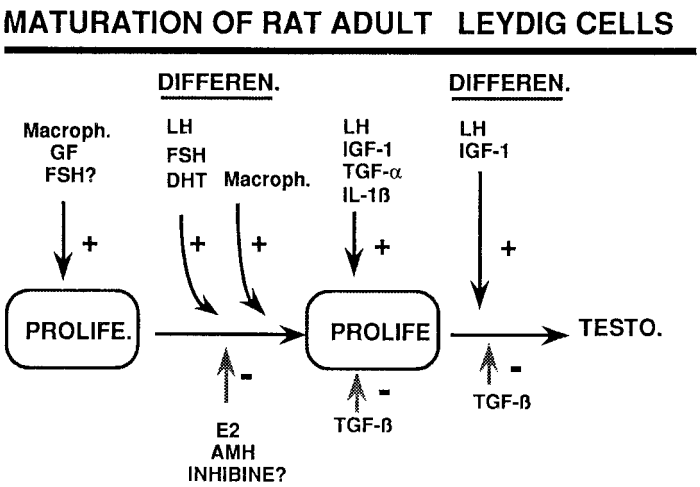
Rat

In the rat, adult Leydig cells are not derived from pre-existing fetal Leydig cells, but from undifferentiated precursor cells. Postnatal development of Leydig cells begins at the stem cell stage (reviewed in Ge *et al*. 1996). These cells proliferate neonatally, doubling approximately every 7 days, to give a population of spindle-shaped undifferentiated cells named 'mesenchymal-like' cells (Hardy *et al*. 1989, Vergowen *et al*. 1991). By day 14 postpartum these cells become committed to the Leydig cell linkage and transformed into progenitor Leydig cells (Fig. 2). Like the stem cells, they are spindle-shaped but are recognizable as members of the Leydig cell lineage, because they express some levels of Leydig cell specific markers, including 3β-hydroxysteroid dehydrogenase (3β-HSD) (Hardy *et al*. 1990), LH receptor and androgen production (Shan & Hardy 1992). By day 28 the Leydig cell progenitors transform from spindle-shape to round cells containing numerous lipid droplets and abundant smooth endoplasmic reticulum (Shan & Hardy 1992). Moreover, the activity of three steroidogenic enzymes, namely P-450 scc, P-450c17 (Shan *et al*. 1993) and 3β-HSD (Dupont *et al*. 1993) sharply increase in immature Leydig cells during days 28 through 56. However, the activity of 17β-HSD, which catalyzes the synthesis of testosterone from androstenedione, does not begin to increase until after day 35 (Eckstein *et al*. 1987). Furthermore, immature Leydig cells produce mostly 5α-reduced androgens, mainly androstane-3α,17β-diol because they express high levels of 5α-reductase and 3α-hydroxysteroid dehydrogenase (3α-HSD) (Murono 1989, Shan *et al*. 1993). Immature Leydig cells divide once between day 28 and day 56 before differentiating into adult Leydig cells.

The transition between immature and adult Leydig cells is characterized by an increase in cell size, volume of smooth endoplasmic reticulum and decline in cytoplasmic lipid droplets. Functionally, mature Leydig cells have higher LH receptor number and increased levels of testosterone biosynthetic enzymes than immature Leydig cells (Shan *et al*. 1993). In contrast, the expression and the activity of the two testosterone metabolizing enzymes, 5α-reductase and 3α-HSD, markedly decline in adult Leydig cells when compared with immature Leydig cells (Shan *et al*. 1993, Viger & Robaire 1995) (Fig. 3).

Adult Leydig cells rarely proliferate (Moore *et al*. 1992) and studies of tritiated thymidine incorporation show that their labeling index is less than 0·1% (Keeney *et al*. 1990). Moreover, the turnover of adult Leydig cells ranges from 142 days to the maximum life span of the animal (Teerds *et al*. 1989*b*). Since the adult Leydig cell population is stable, cell death must be balanced by Leydig cell renewal, probably through proliferation and differentiation of stem cells. In favor of this hypothesis is the fact that intermediate stages of Leydig cell differentiation are observed in adult testes (Hardy *et al*. 1989), and after selective destruction of all adult Leydig cells following administration of the alkylating agent ethane-1,2-dimethyl sulfonate (EDS) the Leydig cell population completely regenerates after 8 to 10 weeks (reviewed in Teerds 1996).

Postnatal proliferation of Leydig cells appears to be controlled by multiple regulatory factors (Fig. 3) (review by Benton *et al*. 1995, Ge *et al*. 1996, Teerds 1996). Although an increased proliferation of precursor Leydig cells has been observed after treatment with exogenous LH or hCG (Christensen & Peacock 1980, Hardy *et al*. 1989, Teerds *et al*. 1989*a*), LH appears to be not essential for proliferation of mesenchymal cells. First, serum LH levels do not rise with the onset of puberty in rats (Dohler & Wuttke 1975). Second, transitory neonatal hypothyroidism induced by treatment



	Precursor L.C	Immature L.C.	Mature L.C.
LHr	+ -	+ +	+ + + +
Anr	+ +	+ + +	+
P450scc	+ -	+ +	+ + + +
3ßHSD	+ -	+ +	+ + + +
P450c17	+ -	+ -	+ + + +
17ßHSD	+ -	+ -	+ + + +
5α–Reductase	-	+ + + +	+
3αHSD	-	+ + +	+

FIGURE 3. Hypothetical model for proliferation and differentiation of Leydig cells in the rat. Anr (androgen receptor); LHr (LH/hCG receptor); E2 (estradiol); macroph. (macrophages); DHT (dihydrotestosterone); testo. (testosterone). The model is a modification of that proposed by Teerds (1996).

with goitrogen propylthiouracil, which permanently suppresses serum LH, increases adult Leydig cell number (Hardy *et al.* 1993, 1996). Third, the mitogenic effect of LH on immature Leydig cells is very small compared with that of several growth factors including insulin-like growth factor-I (IGF-I), TGFα and interleukin-1β (Khan *et al.* 1992*a*,*b*). Fourth, neither chemical suppression of LH nor hypophysectomy inhibits the mesenchymal cell proliferation observed after EDS treatment, but LH is absolutely required for conversion of these precursors into Leydig cells (Teerds *et al.* 1989*a*, Teerds 1996). These latter studies indicate that locally produced growth factors are involved in the regulation of mesenchymal cells, but the nature of these factors is largely unknown.

Some contradictory findings have been reported concerning the effects of follicle-stimulating hormone (FSH), through Sertoli cells, on rat Leydig cell development. FSH treatment of immature hypophysectomized rats not only stimulates seminiferous tubule growth but also induces the formation of adult-type Leydig cells (Kerr & Sharpe 1985, Teerds *et al.* 1989*b*, Vihko *et al.* 1991). Moreover, immature Sertoli cells in response to FSH secrete at least two proteins of 30 kDa and 10 kDa that stimulate proliferation of Leydig cell progenitors (Ojeifo *et al.* 1990, Lamb *et al.* 1991, Wu & Murono 1994). In contrast, FSH treatment of adult hypophysectomized EDS-treated rats did not result in the formation of new Leydig cells (Molenaar *et al.* 1986). One possible explanation for these different observations is that FSH, through Sertoli cells, might have two effects on Leydig cells, namely stimulation of Leydig cell progenitor proliferation and induction of immature Leydig cell differentiation.

In the rat, testicular macrophages are necessary for the development of Leydig cells. Depletion of testicular macrophages in neonatal and immature rats by dichloromethylene diphosphonate prevents the development of adult Leydig cells (Gaytan *et al.* 1994*a*, 1995). Similarly, regeneration of adult Leydig cells after EDS treatment is blocked by depletion of testicular macrophages (Gaytan *et al.* 1994*b*,c). These observations indicate that

macrophages are needed probably together with LH for the initial phases of precursor proliferation as well as for the proliferative activity of immature Leydig cells. The nature of the factors secreted by macrophages and responsible for the proliferation and differentiation of Leydig cell progenitors is still largely unknown. Interleukin-1β may be one of these factors since this is one of the major cytokines secreted by activated macrophages (Dinarello 1994) and *in vitro* this cytokine is a potent mitogen for immature Leydig cells and possibly also for Leydig cell progenitors (Khan *et al*. 1992*b*).

In addition to interleukin 1β, other growth factors, namely IGF-I, TGFα and TGFβ regulate the proliferation of precursor and immature Leydig cells (Khan *et al*. 1992*a,* 1994, Moore & Morris 1993). Both TGFα and IGF-I stimulate the proliferation of precursor Leydig cells isolated from 21-day-old rats, and this effect is potentiated by low concentrations of LH. In contrast, TGFβ inhibits the mitogenic effects of these factors (Khan *et al*. 1994). In addition to their effects on Leydig cell proliferation, two of these factors, IGF-I and TGFβ, regulate in an opposite way, stimulatory and inhibitory respectively, the differentiation of immature into mature Leydig cells. Further evidence of the crucial role of IGF-I on Leydig cell proliferation and differentiation comes from studies of IGF-I knockout mice, showing that Leydig cells of 4-month-old mutants have only reached a stage of morphological and functional differentiation corresponding to days 10-14 postnatal development (Baker *et al*. 1996).

There is some controversy concerning the role of androgens in the development of precursor cells into Leydig cells. One group has reported that dihydrotestosterone in combination with LH can stimulate the differentiation of precursor cells isolated from 21-day-old rat testis (Hardy *et al*. 1990), whereas such an effect was not seen using precursor cells isolated from testes of EDS-treated adult rats (Teerds 1996). Further evidence that androgens might regulate Leydig cell differentiation comes from studies of luteinizing hormone-releasing hormone (LHRH) antagonist in rats and of testicular feminized mouse (Tfm). Administration of LHRH antagonist to 21-day-old rats for 3 weeks prevents the formation of adult Leydig cells, but testosterone replacement restores Leydig cell number and the mRNA levels of LH/hCG receptor and of 3β-HSD (Misro *et al*. 1993, Shan *et al*. 1995). Tfm lacks functional androgen receptor due to a single base deletion in the gene encoding the receptor (Charest *et al*. 1991). Leydig cells of Tfm animals are deficient in P-450c17 and 17β-HSD activities, have low LH/hCG receptor number, low capacity for testosterone production and are unresponsive to LH (O'Shaughnessy & Murphy 1993, Murphy *et al*. 1994). These observations lead to the hypothesis that androgens, directly or indirectly, are required for normal differentiation of adult Leydig cells (Murphy *et al*. 1994). Against this hypothesis is the fact that when testicular slices of Tfm were cultured for 45 h, most Leydig cells became strongly positive for P-450c17 (Le Goascogne *et al*. 1993). This may suggest that local factors rather than the lack of androgen action are responsible for the failure of P-450c17 expression in the adult Tfm mouse. If, in rodents, androgens may play a role in the differentiation of Leydig cell precursors into adult mature Leydig cells, this is not the case in humans, since patients with complete androgen insensitivity have normal levels of plasma testosterone and Leydig cells tend to be hyperplastic (Migeon *et al*. 1994), and mesenchymal cells isolated from patients with androgen insensitivity cultured in the presence of hCG differentiate and produce testosterone (Chemes *et al*. 1992).

In contrast to androgens, estrogens are potent inhibitors of Leydig cell development. A single injection of estradiol to 5-day-old rats inhibits the development of Leydig cells (Dhar & Setty 1976) and administration of estradiol to immature rats inhibits proliferation of Leydig cell progenitors (Abney & Carswell 1986). Similarly, estradiol also prevents regeneration of Leydig cells after EDS treatment (Abney & Myers 1991). These effects of estradiol appear to be direct and not through suppression of gonadotropin secretion, since simultaneous hCG administration was unable to reverse the inhibitory action of estradiol.

There is some indication that gonadal specific peptides may regulate Leydig cell proliferation and differentiation. Thus, AMH-deficient mice (Behringer *et al*. 1994), as well as those in which AMH receptor type II had been inactivated (Mishina *et al*. 1996), developed Leydig cell hyperplasia and in one instance Leydig cell tumor (Behringer *et al*. 1994), suggesting that AMH may inhibit Leydig cell proliferation. Inhibin has been shown to be a tumor suppressor, since 99% of mice of both sexes with deletion of inhibin α subunit gene developed gonadal sex cord-stromal tumors (Sertoli-granulosa tumors) (Matzuk *et al*. 1992, 1996) but Leydig cell number in male mice with gonadal tumor was reduced. Whether this decrease in Leydig cell number is due to tumor development or the absence of inhibin is not quite clear. However, the first hypothesis is more likely since, before tumor development,

spermatogenesis and Leydig cell function appear to be normal.

Figure 3 summarizes the effects of several factors on Leydig cell proliferation and differentiation, as well as the expression of specific genes on precursor, immature and mature Leydig cells.

Human

In humans, the stages in the postnatal development of Leydig cells are less well known than in rodent. Three main periods have been defined: neonatal, infantile or prepubertal, and pubertal (reviewed in Chemes 1996). The neonatal period extends through the first year of life. Just after birth, Leydig cell numbers start to increase to reach a peak at about 3 months of age, which is associated with a peak of plasma testosterone (Forest *et al*. 1973, Fouquet *et al*. 1984). Thereafter, there is a rapid regression of fetal Leydig cells reaching the nadir at the end of the first year (Nistal *et al*. 1986). The infantile period starts at about 1 year of age and extends until the first signs of pubertal development appear. The interstitial space is populated by fusiform or stellate mesenchymal cells. These cells are smaller than adult Leydig cells, have convoluted nuclei and a small amount of smooth endoplasmic reticulum. These features are considered typical of undifferentiated cells (Prince 1984, Chemes *et al*. 1985, 1992, Nistal *et al*. 1986, Chemes 1996). At the beginning of the pubertal period, or following hCG administration during childhood, these mesenchymal cells proliferate and start to differentiate into adult-type Leydig cells. This process of differentiation involves the onset and marked increase of the smooth endoplasmic reticulum and steroid-type mitochondria, and appearance of crystals of Reinke as well as typical changes in nuclear morphology (Prince 1984, Chemes 1996).

If, as indicated above, the functional differentiation of Leydig cells at the very beginning of fetal life appears to be LH/hCG independent, the proliferation and differentiation of Leydig cell precursors after the 10th week of gestation and postnatally are LH/hCG dependent. The evidence for this is fourfold: first, absence of Leydig cells in one patient with male hypogonadism due to a point mutation in the coding sequence of LHβ gene that eliminates the ability of the hormone to bind to its receptor (Weiss *et al*. 1992); second, Leydig cell agenesis in patients having inactivating mutations of LH receptor (Kremer *et al*. 1995, Laue *et al*. 1995, 1996, Latronico *et al*. 1996); third, proliferation and differentiation of Leydig cells during childhood following hCG administration (Chemes *et al*. 1985, Chemes 1996); and fourth, marked morphological and functional differentiation of Leydig cells in the syndrome of familial male precocious puberty due to activating mutation of LH/hCG receptor (reviewed in Shenker 1995).

REGULATION OF LEYDIG CELL FUNCTION

Normal function of the testis has long been recognized to be dependent on the pituitary-synthesized gonadotropins. Notwithstanding these requirements for gonadotropins, the anatomical arrangement of the testis with two compartments, the interstitial tissue and the avascular seminiferous tubules, separated by the blood–testis barrier, points to an active interaction between different testicular cells. Indeed, many data over the past few years clearly indicate that a subtle regulation of testicular function can be locally controlled. However, it should be emphasized that the local regulation acts in conjunction with gonadotropins and very often depends upon these hormones. Four recent articles (Skinner 1991, Ackland *et al*. 1992, Sharpe 1993, Saez 1994) and several chapters of a book (Payne *et al*. 1996) have extensively reviewed this aspect. Therefore, in the present review, only some aspects concerning the regulation of Leydig cell function will be discussed.

Endocrine regulation

LH/hCG is the main hormone which, under physiological conditions, controls Leydig cell function via its specific receptor which is coupled to both adenylate cyclase and phospholipase C pathways (Gudermann *et al*. 1992, Segaloff & Ascoli 1993, Cooke 1996). However, under physiological conditions, most of the effects of LH/hCG on Leydig cells are exerted predominantly through cAMP-mediated events (Saez 1994). Exposure of Leydig cells to LH/hCG causes two types of response. The first, observed within the first minute, is a sharp increase in cAMP and steroid production. This acute steroidogenic effect, which is mainly or exclusively mediated by cAMP, involves translocation of cholesterol from the cytosol to the inner mitochondrial membrane, is sensitive to protein synthesis inhibitors, but does not require RNA synthesis. Several protein candidates have been postulated to be involved in this first and rate-limiting step of steroidogenesis: sterol carrier protein 2, steroidogenesis activating polypeptide, peripheral benzotrazepine receptor and steroidogenic acute regulatory (StAR) protein (reviewed in Papadopoulos 1993, Saez 1994, Stocco 1996). Although the role of each of these proteins, as well

as their mechanisms of action, is still unclear, StAR represents the more attractive candidate from the transfer of cholesterol from cytosol to inner mitochondrial membrane, induced in most steroidogenic tissues (except placenta and brain) by steroidogenic hormones. In favor of this crucial role of StAR, is the fact that in lipoid congenital adrenal hyperplasia, a lethal disease which results from a complete inability to synthesize any steroid (reviewed in New *et al*. 1989), a nonsense mutation of StAR gene has been identified (Lin *et al*. 1995).

The second type of response is the long-term trophic effect of the hormone on Leydig cell structure and function. In addition, LH/hCG can induce an immediate early response, in particular the expression of nuclear proto-oncogenes of the *fos* and *jun* family (Czerwiec *et al*. 1989, Hall *et al*. 1991). Although the role of these transcriptional factors in the long-term effect of LH/hCG is unclear, it has been postulated that they might be the link between the hormone–membrane receptor interaction and the long-term trophic effects (Angel & Karin 1991). The crucial role of LH/hCG on Leydig cell structure and function has been demonstrated by both *in vivo* and *in vitro* studies (reviewed in Saez 1994, Payne & O'Shaughnessy 1996). Thus, in rats, hypophysectomy, suppression of gonadotropins by steroid administration, or neutralization of LH or LHRH by specific antibodies causes Leydig cell atrophy and loss of cytoplasmic smooth endoplasmic reticulum, steroidogenic enzyme activity (in particular P-450c17 and P-450 scc), LH/hCG receptor per Leydig cell, and the ability to secrete testosterone in response to LH/hCG. Treatment of LH-deprived rats with LH or hCG restores, at least partially, the structure and function of Leydig cells (Wing *et al*. 1985, Keeney *et al*. 1988, Teerds *et al*. 1989*a*,*b*, Russell *et al*. 1992). Similarly, treatment of the hypogonadal (hpg) mouse with daily injections of LH produces a marked increase in most of the steroidogenic enzyme activity (O'Shaughnessy 1991). In the intact rat, daily administration of low doses of hCG caused an increase in both 3β-HSD and P-450c17 activities and an increase in *in vitro* basal and hCG-stimulated testosterone production (O'Shaughnessy & Payne 1982). In contrast, a single high dose of hCG or LH resulted in a decrease in LH receptor number and mRNA, a decrease in hCG-induced testosterone production as well as a marked decrease in P-450c17 activity (Cigorraga *et al*. 1978, Saez *et al*. 1978, Chasalow *et al*. 1979, O'Shaughnessy & Payne 1982, LaPolt *et al*. 1991). However, after several days, the number of LH receptors, the P-450c17 activity and the steroidogenic capacity of Leydig cells from both intact and hypophysectomized hCG-treated rats were higher than those of the corresponding controls. Taken together, all the available data indicate that, at low physiological doses, LH/hCG have a positive action on the expression of genes encoding for several Leydig cell specific functions, whereas at high doses, the long-term trophic effects are preceded by a desensitization period. These double and opposite effects of hCG are also present in humans (Saez & Forest 1979).

Many *in vitro* studies using several Leydig cell types have confirmed and extended the above results (reviewed in Payne *et al*. 1992, Saez 1994, Payne & O'Shaughnessy 1996). The main conclusions from both *in vivo* and *in vitro* studies are the following. (1) LH/hCG down regulates its own receptors through at least three mechanisms: internalization–degradation of the hormone–receptor complex (Lloyd & Ascoli 1983, Habberfield *et al*. 1986, Bernier *et al*. 1987), inhibition of LH/hCG gene transcription (Wang *et al*. 1991, Chuzel *et al*. 1995) and increased degradation of LH/hCG mRNA (Lu *et al*. 1993, Chuzel *et al*. 1995). The relative importance of each of these processes in the regulation of LH/hCG receptor depends on the receptor itself and on the type of Leydig cells, which may in turn have species-specific properties. (2) LH/hCG increases the mRNA, protein and activity of P-450 scc, 3β-HSD and P-450c17. However, whereas LH/hCG is absolutely required for P-450c17, the expression of both P-450 scc and 3β-HSD continues in the absence of the hormone (Hales & Payne 1989, Payne & Sha 1991, Keeney & Mason 1992, Clark *et al*. 1996). Although far less studied, it appears that LH/hCG also regulates the other two enzymes of the steroidogenic pathway, namely, 17β-HSD (O'Shaughnessy 1991) and P-450 aromatase (Canick *et al*. 1979, Valladares & Payne 1981, Saez *et al*. 1989). The effects of LH/hCG in the two testosterone metabolizing hormones, 5α-reductase and 3α-HSD, appear more complex. The immature rat testis, between the ages of 20 and 40 days postpartum, secretes mainly 5α-reduced androgens, primarily in the form of 5α-androstan-3α,17β-diol. This pattern of secretion results from low 17β-HSD activity and, more importantly, high activity levels of 5α-reductase activity and 3α-HSD. Recent studies have shown that in the rat testis only type 1 5α-reductase is expressed, and that the peak of expression of both mRNA and protein is between days 21 and 28. Thereafter, both decline rapidly and remain low at least until day 90 (Viger & Robaire 1995). This decline corresponds to the period of transformation of immature to mature Leydig cells. Previous studies have also shown that 5α-reductase

TABLE 4. Endocrine regulation of Leydig cells

	LH/hCG$_R$	P-450scc	P-450 C17	3β-HSD	17β-HSD	hCG-induced testosterone	Reference
Hormone							
LH/hCG	a↑ b↓	↑	↑	↑	↑	↑	See text
FSH	↑	↑	↑	↑	↑	↑	See text
Prolactin	↑	c↑	—	—	—	c↑	Saez & Lejeune 1996
Glucocorticoids	↓	↓	↓	↓	—	↓	Gao *et al*. 1996, Saez & Lejeune 1996
Androgens	d↑	—	d↑	d↑	—	d↑	Shan *et al*. 1995
	e↓	—	e↓	e↓	—	e↓	Payne & O'Shaughnessy 1996, Saez & LeJeune 1996
Calcitonin	—	—	—	—	—	f↑ or g↓	Nakhla *et al*. 1989, Wang *et al*. 1994

a, at low pulsatile concentrations; b, at high continuous concentrations; c, only in immature hypophysectomized rats; d, rat precursor Leydig cells; e, Leydig cells from hypophysectomized adult rat or adult mouse; f, *in vitro*; g, *in vivo*.

activity markedly decreases following hypophysectomy of 21-day-old rats, and that a twice daily injection of LH caused a sharp increase in 5α-reductase activity (Murono & Payne 1976). Similarly, LH increases 5α-reductase activity in hpg mice (O'Shaughnessy 1991). Thus, LH appears to be required for the expression of 5α-reductase in immature Leydig cells. However, since the conversion of immature to mature Leydig cells is LH dependent and, as indicated above, this conversion is associated with a decrease of 5α-reductase expression, LH directly or indirectly appears to reduce the expression of 5α-reductase in adult rat Leydig cells. These double and opposite effects of LH are also probably effective on the regulation of 3α-HSD, since its mRNA levels and activity are higher in precursor and immature than in adult Leydig cells (Shan *et al*. 1993) and since in LH-suppressed immature rats, LH administration significantly increases 3α-HSD mRNA levels in the testis (Shan *et al*. 1995).

In addition to LH and FSH (see below) other endocrine factors have been reported to be able to regulate Leydig cell function. Their effects have been reviewed recently (Saez & Lejeune 1996) and are summarized in Table 4. It must be emphasized that for most of them, the effects have been demonstrated using *in vitro* systems, and that the effects reported are moderate. Thus, except for prolactin and androgens in rodents and for glucocorticoids in all species studied, the physiological role of the other factors on Leydig cell function is of doubtful significance.

Sertoli–Leydig cell interaction

There is a substantial body of evidence to show that Sertoli cells exert a paracrine role on Leydig cell number and function (reviewed in Sharpe 1993, Saez 1994). This evidence derives from several experimental approaches which are summarized here.

Historically, the first evidence that FSH through Sertoli cells modulates Leydig cell function was afforded by Johnson & Ewing (1971), who reported that FSH enhanced testosterone production significantly by perfused rabbit testes exposed to maximal concentrations of LH, but had no effect alone. In support of the involvement of FSH on Leydig cell functions, is also the close correlation between serum FSH levels and the steroidogenic response of Leydig cells to LH/hCG during sexual maturation in both the human (Sizonenko *et al*. 1973) and rat (Odell & Swerdloff 1976). Thereafter, numerous experimental results, obtained using both *in vivo* and *in vitro* models, have confirmed that indeed FSH, indirectly through Sertoli cells, modulates Leydig cell function.

Two *in vivo* models have been used to investigate the effects of FSH on Leydig cells, the hypophysectomized immature rat (Odell & Swerdloff 1976) and the hpg mouse which has undetectable plasma levels of both LH and FSH due to a deletion in the gene encoding for gonadotropin-releasing hormone (GnRH) (Mason *et al*. 1986). Treatment of immature hypophysectomized rats with highly purified pituitary FSH (Teerds *et al*. 1989*a*,*b*, Vihko *et al*. 1991) or recombinant human FSH (hFSH) (Vihko *et al*. 1991, Russell *et al*. 1993, Matikainen *et al*. 1994) not only stimulates seminiferous tubule growth but also induces Leydig hypertrophy and hyperplasia and increases LH receptor number and mRNA and the *in vitro* steroidogenic response to hCG. Similarly, FSH treatment of adult hpg mice for 10 days markedly enhanced the steroidogenic responsiveness both *in vivo* and *in vitro* to hCG,

and this was associated with an enhanced activity of cholesterol side-chain cleavage, P-450c17, 17β-HSD and, to a lesser extent, of 3β-HSD (O'Shaughnessy *et al*. 1992). Similar results have been obtained using hypophysectomized Golden hamsters (Klemcke *et al*. 1986).

Second, experimental disruption of spermatogenesis, induced by X-irradiation, cryptorchidism, vitamin A deficiency, efferent ligation or heat treatment, resulted in morphological and functional changes of Leydig cells (reviewed in Sharpe 1993, Saez 1994). In most of these experimental conditions, there was an increase in the plasma levels of LH and FSH, but the Leydig cell changes were not due to these increments, since local implantation of anti-androgens in the testis produced local areas of damage of the seminiferous epithelium and, adjacent to these areas but not in unaffected areas of the same testis, there were the morphological modifications of the interstitial cells described above (Aoki & Fawcett 1978). These results clearly indicate the existence of a local mechanism of controlling Leydig cell function.

Third, possibly more convincing are the many studies which have shown that co-culture of Sertoli cells with Leydig cells modulates the steroidogenic responsiveness of Leydig cells. Co-culture of Leydig cells with Sertoli cells isolated from immature pig testis, enhances hCG-stimulated testosterone production when compared with the response of Leydig cells cultured alone. Pretreatment of co-cultures with FSH further enhances the steroidogenic capacity of Leydig cells and induces a significant increase in the number of hCG receptors (Tabone *et al*. 1984, Benahmed *et al*. 1985, Reventos *et al*. 1989, Saez *et al*. 1989). These functional changes of Leydig cells were associated with a hypertrophy of the smooth endoplasmic reticulum and an increase in the number of cytoplasmic lipid droplets, which correlated with the increased steroidogenic activity. Similarly, co-culture of immature rat Sertoli cells with immature rat Leydig cells (Verhoeven & Cailleau 1990) or with rat Leydig cell tumor H-540 cells (Verhoeven & Cailleau 1991), either in the same dish or in a two-chamber system, enhances basal and LH- or dibutyryl cAMP-stimulated steroid production and these effects are significantly augmented by pretreating the co-culture with FSH. More recently (Lejeune *et al*. 1993), it has also been shown that co-culture of adult human Leydig cells with human Sertoli cells, not only prevents the decline in the steroidogenic capacity observed when Leydig cells are cultured alone, but greatly enhances their capacity to produce testosterone. This increased steroidogenic capacity of the Leydig cells co-cultured with Sertoli cells is associated with an increase in the mRNA levels of P-450 scc, P-450c17 and 3β-HSD (H Lejeune and J M Saez, unpublished observations).

Finally, the stimulatory effect of FSH on Leydig cells has also been demonstrated using rat fetal testis explants (Lecerf *et al*. 1993). In this *in vitro* system an hFSH preparation contaminated with small amounts of LH induced basal and acute LH-stimulated testosterone production. The specificity of the FSH effects was demonstrated by the fact that specific anti-hFSHβ antibodies, but not anti-hLHβ antibodies, blocked the effect of hFSH, and by the fact that recombinant hFSH produced similar effects.

Since the current consensus is that Sertoli cells are the major target for FSH in the testis (Griswold 1993), it is likely that most of the *in vivo* and *in vitro* effects of FSH are mediated through Sertoli cell secreted production. In favor of this hypothesis is the fact that conditioned medium from rat (Verhoeven & Cailleau 1985, Carreau *et al*. 1988, Ojeifo *et al*. 1990), human (Verhoeven & Cailleau 1987) and pig (Perrard-Sapori *et al*. 1987) Sertoli cells modulates Leydig cell functions, and the acute stimulatory effect on testosterone production was enhanced when Sertoli cells were pretreated with FSH (Verhoeven & Cailleau 1985, 1987, Perrard-Sapori *et al*. 1987). Interestingly, when rat Sertoli cells were cultured in a two-chamber system, more than 80% of the steroidogenic factor(s) were found in the basal compartment, indicating that the factor(s) was secreted in a polarized manner (Onoda *et al*. 1991). Moreover, the addition of pachytene spermatocytes or pachytene spermatocyte proteins to the apical compartment of the chambers, inhibited by 85% the basally directed Sertoli cell secretion of the steroidogenic factor(s). Recently, the FSH-induced factor responsible for the acute stimulation of Leydig cell steroidogenesis (steroidogenesis-stimulating protein) has been purified and isolated and identified as an inhibitor of metalloproteinase-1 (Boujrad *et al*. 1995). In addition to these acute steroidogenic effects, conditioned medium from Sertoli cells also has a long-term effect on Leydig cells, the nature and the intensity of which depend upon the conditions in which Sertoli cells are cultured. Thus, conditioned medium from Sertoli cells of several species cultured in the absence of FSH (Papadopoulos *et al*. 1987, Perrard-Sapori *et al*. 1987), had an inhibitory action on several parameters of Leydig cell function, whereas medium from Sertoli cells treated with FSH (Perrard-Sapori *et al*. 1987) had the opposite effect. Moreover, as indicated before, immature Sertoli cells in response to FSH secrete at least two proteins

of 30 kDa and 10 kDa that stimulate proliferation of Leydig cells, effects that were more pronounced on Leydig cell progenitors (Ojeifo *et al*. 1990, Lamb *et al*. 1991, Wu & Murono 1994).

Further evidence in favor of the positive effects of FSH on Leydig cell function was afforded by the study of a hypophysectomized man who, despite undetectable levels of serum gonadotropins, had normal testis volume, almost normal spermatogenesis and low but detectable levels of serum testosterone. These surprising clinical data were due to a heterozygous activating mutation of FSH receptor (Gromoll *et al*. 1996). These findings therefore indicate that FSH is not only important for Sertoli cell function, but that the hormone in the absence of LH might be able to maintain some Leydig cell function.

Although all the above *in vivo* and *in vitro* findings strongly suggest an important role of FSH on Leydig cell development and function, two recent studies question this hypothesis. Thus, inactivation of FSHβ subunit gene produces fertile male mice with apparent normal development and function of Leydig cells, although with moderate reduction of testicular volume. In contrast, homozygous females were infertile with complete arrest of follicular maturation (Kumar *et al*. 1997). Similarly, men homozygous for an inactivating mutation of FSH receptor had small testes, normal plasma testosterone levels and variable degrees of spermatogenesis failure, but two out of five had children (Tapanainen *et al*. 1997). In contrast, homozygous females for the same mutation were infertile with arrest of follicular maturation (Aittomöki *et al*. 1995, 1996). Both results clearly indicate that whereas FSH is absolutely required for normal ovarian function, it is not required for normal development and function of Leydig cells.

Interaction of Leydig cells with other testicular cells

Theoretically, Leydig cell function can be regulated by the other cells present in the interstitial compartment, namely peritubular myoid cells and macrophages. Although there is strong evidence indicating an active cooperation between Sertoli cells and peritubular cells (reviewed in Skinner 1991), there are few data concerning the interaction between Leydig cells and peritubular cells. There is no evidence for a direct effect of peritubular cells or their secreted proteins on Leydig cell function (Risbridger & Skinner 1992). However, peritubular cell secreted proteins, in particular P-Mod-S, regulate Sertoli cell function (Norton & Skinner 1989). Thus, peritubular cells might regulate Leydig cell function, indirectly, through Sertoli cells.

The interaction between Leydig cells and macrophages as well as the factors involved in this interaction have been recently reviewed (Hales 1996). As indicated above, in the rat testicular macrophages are needed for the initial phases of precursor proliferation as well as for the proliferative activity of immature Leydig cells, but not for the maintenance of mature Leydig cell functions (Gaytan *et al*. 1994*a*, 1995). Further evidence for the role of testicular macrophages on testicular development and function came from studies of the osteopetrotic (op/op) mouse characterized by an autosomal mutation in colony stimulating factor 1 resulting in a deficiency of both macrophages and osteoclasts (Pollard & Stanley 1996). These mice had low plasma testosterone levels, and *in vitro* LH- and 22(R)-hydroxycholesterol-stimulated testosterone productions were reduced compared with Leydig cells isolated from op/+ mice (Cohen *et al*. 1996). In addition, there are some findings indicating that testicular macrophages may be involved in the effect of FSH on Leydig cells. First, unilateral depletion of testicular macrophages in hypophysectomized 28-day-old rats abolishes the stimulatory effect of FSH on Leydig cell number only in macrophage-depleted testis (Gaytan *et al*. 1995). Second, testicular macrophages bind specifically ^{125}I-FSH and FSH stimulation induces increased lactate and cAMP production by these cells (Yee & Hutson 1985*a*,*b*). More importantly, conditioned medium from testicular macrophages was able to stimulate both basal and LH-stimulated testosterone production by isolated rat Leydig cells (Yee & Hutson 1985*c*). Conditioned medium from macrophages previously treated with FSH was twice as potent as conditioned medium from untreated macrophages. In contrast, another group (Lombard-Vignon *et al*. 1991) reported that macrophage-conditioned medium from control or FSH-treated rats inhibited Leydig cell testosterone production. More recently (Mayerhofer *et al*. 1992*b*), it has been shown that macrophages from active but not from regressed testes of the Siberian hamster responded to FSH by an increased lactate production, suggesting the presence of functional FSH on macrophages. However, whereas conditioned medium from testicular macrophages cultured without FSH had no effect on testosterone production by slides of testicular tissue, an inhibitory effect was observed with conditioned medium from macrophages pretreated with FSH. Whether these discrepancies are due to differences in the species or in the *in vitro* system used

TABLE 5. Criteria required to establish a paracrine/autocrine role for any factor

1.	Presence of receptors and biological action on local cells
2.	Local secretion regulated by physiological signals
3.	Blockade of the factor or its receptor by antibody, antagonist or antisense oligodeoxynucleotides, must modify the function of local cells
4.	Systemic supply of the factor does not explain the regulation

remains to be elucidated. The only certainty is that further studies are needed to clarify the potential role of testicular macrophages on mature Leydig cell functions.

Regulation of Leydig cells by locally produced factors

An approach used to identify locally produced factors able to regulate Leydig function has been to study the effects of known factors on Leydig cell function and to determine whether these factors are produced within the testis. By using these approaches many potential regulatory molecules have been demonstrated to be present in the testis and/or to act on Leydig cells. However, relatively few of these molecules have fulfilled the criteria needed to establish that a molecule found in any tissue might play a local regulatory role (Table 5).

It is pointless to review all the data concerning the testicular production of a large number of these factors and their potential effects on Leydig cell function. Some of these data are summarized in Table 6 and the reader is referred to other recent reviews in which this topic has been covered in more detail (Skinner 1991, Ackland *et al.* 1992, Sharpe 1993, Saez 1994, Hales 1996, Lin 1996, Saez & Lejeune 1996). This review will emphasize factors for which the three criteria, defined above, to be considered as paracrine/autocrine factor(s) have been fulfilled.

IGF-I

There is strong evidence derived from both *in vivo* and *in vitro* studies that both circulating and testicular produced IGF-I may be involved in the proliferation, differentiation and function of Leydig cells. The evidence from *in vivo* studies is the following. (1) In humans, isolated GH deficiency (Kulin *et al.* 1981) or GH resistance, as in the case of Laron syndrome (Laron 1984), is associated with micropenis, suggesting a decreased fetal Leydig function during the second half of pregnancy,

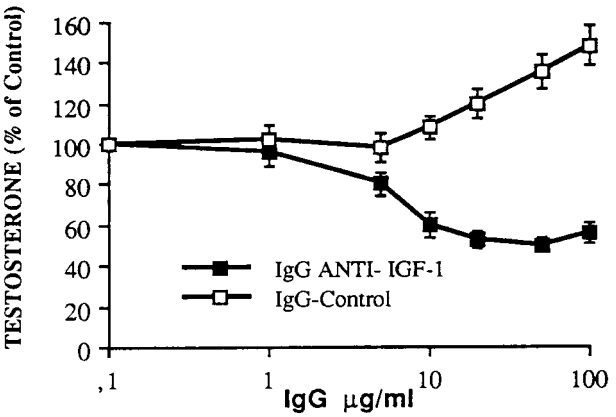


FIGURE 4. Effect of increasing concentrations of IgG prepared from IGF-I antiserum (■) or non-immune serum (□) on Leydig cells. Cells were cultured for 2 days with the indicated concentrations of IgG. At the end of this period, cells were incubated for 24 h with hCG (10^{-9} M) and the testosterone in the medium measured. The results, expressed as a percent of control values, are means ± S.E.M. of three experiments, each in triplicate.

delayed puberty and poor response to exogenous hCG (Kulin *et al.* 1981) which, in the case of GH deficiency, is very often improved following treatment with GH (Rivarola *et al.* 1972, Kulin *et al.* 1981). (2) Administration of GH but also of IGF-I to Snell dwarf mice for 7 days increases the number of testicular LH receptors and the steroidogenic response to hCG (Chatelain *et al.* 1991). (3) The most strong evidence that IGF-I is crucial in the development and function of Leydig cells came from studies of IGF-I gene knockout mice (Baker *et al.* 1996). In these animals, the testes were reduced in size more than expected from the degree of dwarfism, the number and the volume of Leydig cells were markedly reduced, as well as plasma testosterone levels and the *in vitro* basal and LH-stimulated testosterone production by testicular slices were impaired.

In vitro studies have shown that IGF-I fulfilled the first three criteria to be considered as a paracrine/autocrine factor. This topic has been extensively reviewed (Saez 1994, Lin 1996) and we emphasize here the main data: (1) presence of IGF type I receptors in Leydig cells of several species, and stimulatory effect of the peptide in the transcription rate of LH/hCG receptor and several steroidogenic enzymes (Chuzel *et al.* 1996); (2) expression of IGF-I mRNA and protein by somatic testicular cells; and (3) inhibition of rat (Verhoeven & Cailleau 1990) and pig (Fig. 4) Leydig cell steroidogenic response to LH/hCG

when they are cultured in the presence of an IGF-I antiserum.

Taking together the *in vivo* and *in vitro* results, it appears that IGF-I is one of the factors for which there is convincing evidence to postulate that, in addition to its endocrine role, this factor plays a paracrine/autocrine role in the regulation of testicular functions.

TGFβ

Many *in vitro* studies have demonstrated that TGFβ fulfills the first two criteria to be considered as a paracrine/autocrine factor of Leydig cells (reviewed in Saez 1994, Saez & Lejeune 1996): (1) presence of TGFβ receptors in testicular cells and potent inhibitory effects of this peptide on the expression of LH/hCG receptor and P-450c17 gene (Chuzel *et al*. 1996); and (2) expression of TGFβ mRNAs and proteins by testicular cells. Recently we have demonstrated that $TGF\beta_1$ fulfilled the third criterion to be considered as a paracrine/autocrine factor in Leydig cells by using an antisense approach. Transfection of these cells with an antisense oligodeoxynucleotide completely blocked $TGF\beta_1$ synthesis, and this was associated with an increase of LH/hCG receptors and P-450c17 mRNAs (C Le Roy, P Leduque, Y Li, J M Saez & D Langlois, unpublished observations). This antisense approach has already been used in other models to demonstrate the paracrine/autocrine role of TGFβ1 (Wang *et al*. 1995, Jachimczak *et al*. 1996, Le Roy *et al*. 1996, Turley *et al*. 1996).

Despite the above findings clearly demonstrating the autocrine/paracrine role of $TGF\beta_1$ *in vitro*, recent targeting inactivation of $TGF\beta_1$ and one of its receptors has not allowed confirmation of the role of these peptides in Leydig cell development. Thus, normal male phenotype at birth has been reported in mice in which $TGF\beta_1$ (Shull *et al*. 1992, Kulkarni *et al*. 1993, Dickson *et al*. 1995) or $TGF\beta_3$ (Kaartinen *et al*. 1995, Proetzel *et al*. 1995) were inactivated. Moreover, inactivation of TGFβ type II, obligatory mediator of all isoforms of TGFβs, resulted in embryonic lethality around 10·5 dpc before testicular organogenesis (Oshima *et al*. 1996).

Other potential paracrine/autocrine factors

Table 6 enumerates other factors which have been reported either to be produced within the testis and/or to act *in vitro* on Leydig cells. However, for none of them, except IGF-I and TGFβ, has the third criterion to be considered as a paracrine/autocrine factor been demonstrated. Moreover, very often their secretion and their action on Leydig cells have only been shown in the rat, but some of the results observed in this species cannot be extrapolated to others, i.e. rat Leydig cells contain GnRH specific receptor and this peptide acutely stimulates testosterone production, but mouse, pig and human Leydig cells lack GnRH receptors. In addition, it remains to be proven that all the *in vitro* data can be extrapolated to the *in vivo* situation. In this respect, recent findings observed in transgenic animals or in humans with mutations affecting some factors or their receptors, are of great interest.

There are many data *in vitro* which can be interpreted as evidence for modulation of Leydig cells by arginine vasopressin (AVP), but the *in vivo* findings in both rat and the human do not support such hypotheses. In the Brattleboro rat in which AVP is mutated (Ivell *et al*. 1986), plasma testosterone levels, as well as the steroidogenic responsiveness to hCG of isolated Leydig cells, were similar to those of control rats (Collu *et al*. 1984). Similarly, in humans with familial autosomal neurogenic diabetes insipidus due to mutation of the vasopressin–neurophysin gene (Ito *et al*. 1991) or with X-linked nephrogenic diabetes insipidus due to mutation of vasopressin type V_2 receptor (Bichet *et al*. 1993), a dysfunction of Leydig cells has not been reported.

Also, in both rodents and humans, angiotensin II (AngII) appears to have no role in the regulation of Leydig cell function *in vivo*. No change in testosterone levels has been reported in patients or rodents with high plasma AngII levels, either before or after treatment with converting enzyme inhibitor or angiotensin-1 (AT_1) receptor antagonist. Moreover, in transgenic mouse carrying both human renin and human angiotensinogen genes leading to overproduction of AngII, no change in Leydig cell function was reported (Fukamizu *et al*. 1993). Similarly, in mice, inactivation by homologous recombination of angiotensinogen (Tanimoto *et al*. 1994, Smithies & Maeda 1995), AT_1 receptor (Ito *et al*. 1995) or AT_2 receptor (Hein *et al*. 1995, Ichiki *et al*. 1995), no abnormality of testicular function has been reported.

Similarly, although activin and inhibin are specific testicular-produced peptides, and these peptides have been shown to regulate Leydig cell function *in vitro* (reviewed in Risbridger 1996), targeting inactivation of these genes is against a role of these peptides on Leydig cell development and function *in vivo*. Thus, knockout of activin βB subunit, giving mice deficient in activin B, activin AB and inhibin B, results in males with normal reproductive capacity (Vassalli *et al*. 1994). Activin βA-deficient mice develop to term but die within 24 h secondary to multiple craniofacial abnormalities, but without apparent abnormalities of the external or internal genitalia. Similarly, mice deficient in both activins βA and βB, display the

TABLE 6. Main factors produced within the testis and acting in Leydig cells

	Site of production	Evidence	Regulation	Leydig cells	
				Receptor	Effects
Factor					
Steroidogenic stimulatory factor	SC	Protein	↑ FSH	ND	↑ Steroidogenesis
Steroidogenic inhibitory factor(s)	SC	Protein		ND	↓ Differentiated functions
Mitogenic factor(s)	SC	Protein	FSH ↑	−	↑ Leydig cell progenitor proliferation
IGF-I	LC, SC	mRNA, protein	FSH ↑ in SC hCG ↑ in LC	+	↑ differentiated functions
TGFβs	LC, SC, PC	mRNA, protein	FSH ↓ in SC	+	↓ Differentiated functions
EGF/TGFα	LC, SC, GC, PC	mRNA, protein	?	+	↑ Steroidogenesis ↓ Differentiated functions
FGF	LC, SC, GC, PC	mRNA, protein	FSH ↑ in SC	+	↓ Differentiated functions
PDGF	LC	Protein	↑ hCG in LC	+	↓ Differentiated functions
Inhibin/activin	LC, SC	mRNA, protein	FSH ↑ in SC hCG ↑ in LC	ND	Inhibin ↑ steroidogenesis Activin ↓ steroidogenesis
Interleukin-1	LC, SC, M	mRNA, protein	LPS ↑ in SC hCG and LPS ↑ in LC	+	↓ Differentiated functions
Interleukin-2	L	mRNA, protein	?	+	↓ Differentiated functions
Interferon (α, γ)	L	mRNA, protein	?	?	↓ Differentiated functions
TNF-α	GC	mRNA	?	ND	Stimulatory: rat Inhibitory: pig, mouse
LHRH	SC	LHRH-like protein	?	+	Acute ↑ steroidogenesis only in rat
GHRH	LC, GC	mRNA, protein	hCG ↑ in LC	ND	Stimulatory or no effect
CRF	LC	mRNA, protein	hCG ↑ in LC	+	↓ LH-stimulated steroidogenesis in rat ↑ LH-stimulated steroidogenesis in mouse
AVP	LC, SC	mRNA, protein	—	+	Acute ↑ steroidogenesis ↓ Differentiated functions
Oxytocin	LC, SC	mRNA, protein	LH ↑ LC	+	↓ Differentiated functions
ANF	Testis	mRNA, protein	?	+	Stimulatory mouse, inhibitory MA-10 cells No effect rat, human
CNF	LC	mRNA, protein	?	+	?
A-II	LC	Protein		+	↓ Inhibitory
Endothelin	SC	mRNA, protein	FSH ↓ in SC	+	↑ Steroidogenesis
NO	M	—	—	−	↓ LH-stimulated steroidogenesis
NRY	LC, SC	mRNA, protein	FSH ↑ SC, LH ↑ LC	?	?

ANF: atrial natriuretic factor; AVP: vasopressin; CNF: C-type natriuretic factor; CRF: corticotropin-releasing factor; EGF: epidermal growth factor; FGF: fibroblast growth factor; GC: germ cell; GHRH: growth hormone releasing factor; IGF-I: insulin-like growth factor I; L: lymphocyte; LC: Leydig cell; M: macrophage; ND: not determined; NO: nitric oxide; PC: peritubular cell; PDGF: platelet-derived growth factor; SC: Sertoli cell; TGFβ or α: transforming growth factor β or α; TNF-α: tumor necrosis factor α; NRY: neuropeptide Y.
References for Table 6: References before 1994 can be found in Ackland *et al*. 1992, Hales 1996, Lin 1996, Saez 1994, Saez & Lejeune 1996. More recent references concern the effects of nitric oxide (Del Punta *et al*. 1996), C-type natriuretic factor (Middendorff *et al*. 1996), CRF (Huang *et al*. 1995) expression and regulation of neuropeptide Y (Kanzaki *et al*. 1996) and expression of EGF, TGFα and their receptors during testicular development (Caussanel *et al*. 1996).

defects of both activins βA and βB mutant mice, but no additional defects (Matzuk *et al*. 1995*b*). Only inactivation of one of the two activin type II receptors caused a marked reduction in testicular weight associated with a delay in fertility of about 3 weeks compared with heterozygous mice for such mutation (Matzuk *et al*. 1995*a*). However, this abnormality is probably secondary to the very low plasma FSH levels. Finally, homozygous α-inhibin-deficient mice were initially healthy and had normal external genitalia, but were infertile (Matzuk *et al*. 1992). This was due to the development of gonadal sex cord-stromal tumors (granulosa/Sertoli cell tumors) in both sexes as early as 4 weeks of age. However, spermatogenesis, as well as the number of Leydig cells, was normal in male from 5–7 weeks,

but a regression of both parameters occurred in parallel with the enlargement of the tumor mass. Interestingly, inhibin deficient mice have very high plasma levels of both activin A and activin B (Matzuk *et al*. 1994), but these high levels of activins are not responsible for the gonadal sex cord-stromal tumor development (Coerver *et al*. 1996, Matzuk *et al*. 1996). Thus, the clear-cut conclusion for all the above studies is that inhibins function as tumor suppressors in both gonads and adrenal cortex (Matzuk *et al*. 1994, 1996), but that inhibins and activins are not important for Leydig cell development and function.

Although many *in vitro* studies have suggested a paracrine/autocrine regulation of Leydig cells by epidermal growth factor (EGF)/TGFα (reviewed in Ackland *et al*. 1992, Saez 1994, Saez & Lejeune 1996), targeted disruption of TGFα (Luetteke *et al*. 1993, Mann *et al*. 1993) or of the EGF receptor (Sibilia & Wagner 1995, Threadgill *et al*. 1995) has not allowed confirmation of such a hypothesis. Thus, mice homozygous for a disrupted TGFα gene are healthy and fertile, the only abnormality was a pronounced waviness of the coat. The phenotype of EGF receptor inactivation was dependent on genetic background, causing either peri-implantation death, death at mid-gestation due to placental defects, or normality at birth followed by progressive reduction in weight and wasting. The latter group lived for up to 3 weeks and showed abnormalities in skin, kidney, brain, liver and gastrointestinal tract (Threadgill *et al*. 1995). However, because of the early death, the fertility of these mutants is unknown.

Also many *in vitro* studies have suggested that fibroblast growth factors (FGFs) regulate Leydig cell function (reviewed in Saez 1994, Saez & Lejeune 1996), but their physiological role *in vivo* has not yet been demonstrated. Since *in vitro* studies have shown that the long-term effects of FGF on Leydig cells are inhibitory, one would expect that activating mutations of FGF receptors would cause inhibition of Leydig cell function. This prediction has not been confirmed, at least in humans, since no apparent abnormalities in testicular function have been reported in patients with Pfeiffer syndrome (Muenke *et al*. 1994), Crouzon syndrome (Reardon *et al*. 1994) and achondroplasia (Shiang *et al*. 1994) who had an activating mutation of FGF receptors 1, 2 and 3 respectively.

Oxytocin has been reported to be either inhibitory (Adashi *et al*. 1987) or stimulatory (Tahri-Joutei & Pointis 1989, Frayne & Nicholson 1995) on isolated Leydig cell testosterone production. In contrast, *in vivo* supraphysiological levels of oxytocin released from oxytocin-filled testicular implants reduced both serum and testicular testosterone levels (Nicholson *et al*. 1991). This inhibitory effect was confirmed recently in a transgenic mouse model overexpressing oxytocin in the testis (Ang *et al*. 1994).

CONCLUDING REMARKS

This review provides evidence of the great progress made in the last two decades in our understanding of the origin, development and regulation of Leydig cells owing to progress in cellular and molecular biology. Most of the data concerning the multifactorial regulation of Leydig cells have been generated by *in vitro* studies using isolated cells or co-culture. The advantage of these models is that they have allowed a better definition at the cellular and molecular levels of the secretion and action of many factors. Their weakness is that they destroy the complex and highly organized testicular structure and therefore the multiple cell–cell interactions. Thus, the extrapolation of the *in vitro* findings to the *in vivo* situation requires some controls before some physiological relevance can be assigned to them.

To improve our understanding of Leydig cell development and function, future research needs more sophisticated *in vivo* studies, including: (1) production of transgenic animals overexpressing a factor or its receptor driven by tissue-specific promotor; (2) overexpression of the corresponding antisense mRNA driven by tissue-specific or inducible promotor; and (3) targeted disruption of such factors or receptors.

Finally, since Leydig cells form part of a complex tissue, in which the cross-talk between different testicular cell types appears to be required to allow the testis to fulfill both its endocrine and exocrine functions, the regulation of Leydig cells must be integrated with that of the other somatic cells and germ cells via a short-loop feedback system. Thus, the above approach, overexpression, and knockout should also be used for the other testicular cells.

ACKNOWLEDGEMENTS

We are grateful to Joëlle Bois and Marie-Ange Di Carlo for their secretarial assistance. Part of the work was supported by a grant of Université Claude-Bernard.

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RECEIVED 21 April 1997