

Morphological and functional maturation of Leydig cells: from rodent models to primates

Katja J. Teerds^{1,*} and Ilpo T. Huhtaniemi^{2,3}

¹Human and Animal Physiology, Wageningen University, De Elst 1, 6709 WD, Wageningen, The Netherlands ²Department of Surgery and Cancer, Institute of Reproductive and Developmental Biology, Imperial College London, Hammersmith Campus, Du Cane Road, W12 0NN London, UK ³Department of Physiology, Institute of Biomedicine, University of Turku, Kiinamylynkatu 10, 20520 Turku, Finland

*Correspondence address. E-mail: katja.teerds@wur.nl

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BACKGROUND: Leydig cells (LC) are the sites of testicular androgen production. Development of LC occurs in the testes of most mammalian species as two distinct growth phases, i.e. as fetal and pubertal/adult populations. In primates there are indications of a third neonatal growth phase. LC androgen production begins in embryonic life and is crucial for the intrauterine masculinization of the male fetal genital tract and brain, and continues until birth after which it rapidly declines. A short post-natal phase of LC activity in primates (including human) termed ‘mini-puberty’ precedes the period of juvenile quiescence. The adult population of LC evolves, depending on species, in mid- to late-prepuberty upon reawakening of the hypothalamic–pituitary–testicular axis, and these cells are responsible for testicular androgen production in adult life, which continues with a slight gradual decline until senescence. This review is an updated comparative analysis of the functional and morphological maturation of LC in model species with special reference to rodents and primates.

METHODS: Pubmed, Scopus, Web of Science and Google Scholar databases were searched between December 2012 and October 2014. Studies published in languages other than English or German were excluded, as were data in abstract form only. Studies available on primates were primarily examined and compared with available data from specific animal models with emphasis on rodents.

RESULTS: Expression of different marker genes in rodents provides evidence that at least two distinct progenitor lineages give rise to the fetal LC (FLC) population, one arising from the coelomic epithelium and the other from specialized vascular-associated cells along the gonad-mesonephros border. There is general agreement that the formation and functioning of the FLC population in rodents is gonadotrophin-responsive but not gonadotrophin-dependent. In contrast, although there is in primates some controversy on the role of gonadotrophins in the formation of the FLC population, there is consensus about the essential role of gonadotrophins in testosterone production. Like the FLC population, adult Leydig cells (ALC) in rodents arise from stem cells, which have their origin in the fetal testis. In contrast, in primates the ALC population is thought to originate from FLC, which undergo several cycles of regression and redifferentiation before giving rise to the mature ALC population, as well as from differentiation of stem cells/precursor cells. Despite this difference in origin, both in primates and rodents the formation of the mature and functionally active ALC population is critically dependent on the pituitary gonadotrophin, LH. From studies on rodents considerable knowledge

has emerged on factors that are involved besides LH in the regulation of this developmental process. Whether the same factors also play a role in the development of the mature primate LC population awaits further investigation.

CONCLUSION: Distinct populations of LC develop along the life span of males, including fetal, neonatal (primates) and ALC. Despite differences in the LC lineages of rodents and primates, the end product is a mature population of LC with the main function to provide androgens necessary for the maintenance of spermatogenesis and extra-gonadal androgen actions.

Key words: developmental stage and functionality / Leydig cells / development regulating factors / animal models

Introduction

The main function of Leydig cells (LC) is to produce androgens for the paracrine regulation of spermatogenesis within the testis, and for the various systemic endocrine effects, androgenic and anabolic, outside the testis. LC androgen production begins in embryonic life and is crucial for masculinization of the male fetus. In addition to androgens, fetal Leydig cells (FLC) produce a peptide hormone, insulin like-3 (INSL3) that participates in the induction of testicular descent. Testicular endocrine activity continues until birth, and a few weeks (rodents) or months (primates; mini-puberty) beyond, followed by hormonal quiescence until puberty. During the quiescent period, most FLC disappear or dedifferentiate and will be replaced at mid-prepuberty (rodents) or at puberty (primates), when function of the hypothalamic–pituitary–testicular axis reawakens, by another population of cells, the adult Leydig cells (ALC) (reviewed in Haider, 2004). Although two growth phases of LC are identifiable in most mammalian species, they differ in morphology, regulation, functional features and physiological actions. Such differences are expected and physiologically meaningful, considering the vastly different environments they function in, i.e. *in utero* during the morphogenesis and functional differentiation of sexual organs, and *ex utero* upon their growth, maturation and maintenance of mature functions. For the same reasons, testicular hormones have very different functions at different periods of life. Our purpose is to present an updated review of the salient features in the differentiation, regulation and function of LC along a male's lifespan. We concentrate on two model species, i.e. rodents and primates, and review findings in other species only when relevant. This text is not intended to be an exhaustive review, and we have minimized the systematic presentation of the topic, concentrating on findings not yet found in this context in textbooks and other reviews.

Methods

Most studies on the regulation of LC development in the prenatal and post-natal testis have been performed in model animals, mainly rodents; the information on primates is unfortunately still scarce. The first part of this review therefore focuses on the origin, differentiation and functionality of LC during these periods of life in rodents; data on primates are presented when available. The second part gives an up-to-date overview of our current knowledge on LC development and function in primates with specific emphasis on humans, and the differences with the rodent models. The third part of the review discusses the consequences of aging on LC morphology and functionality in primates and animal models. Only peer-reviewed articles have been cited. Review articles have been used as a reference source and are referred to as such.

Exclusion criteria

Studies published in other languages than English or German are excluded, as are studies of which only the abstracts are available.

Search strategy

Pubmed, Scopus, Google Scholar and Web of Science have been comprehensively searched. Search terms used included combinations of the following terms: fetal Leydig cell development/differentiation, adult Leydig cell development/differentiation, stem Leydig cells, human Leydig cell development/differentiation, primate Leydig cells, Leydig cells and aging, testicular aging, fetal Leydig cell steroidogenesis, adult Leydig cell steroidogenesis, neonatal Leydig cells, Leydig cell progenitors, immature Leydig cells, mature adult Leydig cells.

Results

FLCs

Cellular origin of FLC

Gonadal differentiation starts from a layer of coelomic epithelial cells adjacent to the mesonephros. Upon subsequent differentiation of the undifferentiated gonad into the testis, following expression of the *SRY* gene located on the Y chromosome (Hacker *et al.*, 1995; Sekido and Lovell-Badge, 2013), two distinct compartments will be defined, i.e. the testicular cords and the interstitium. The testicular cords give rise to seminiferous tubules of the adult testis and the interstitium to steroidogenic LC and other less well-defined cells of the interstitial space. The somatic progenitor cells, subsequently giving rise to FLCs and Sertoli cells, start early on expressing steroidogenic factor 1 (*Sf1*) (Chen *et al.*, 2010). Although differentiation of the Sertoli cells within testicular cords is considered the defining step of testicular differentiation, also that of LC is an important milestone of testicular differentiation.

Relatively little is still known about the molecular and cellular diversity within the interstitial space of the embryonic testis. Recent findings of DeFalco *et al.* (2011, 2013) on mice indicated molecular heterogeneity with distinct molecular markers of specific cell types. On the basis of expression of these different marker genes, it was concluded that at least two distinct progenitor lineages give rise to FLC, one arising from the coelomic epithelium and the other from specialized vascular-associated cells along the gonad-mesonephros border. Perivascular cells from the mesonephric border likely represent an interstitial cell precursor lineage. The early interstitial cells are closely associated with invading blood vessels, and complex paracrine cell–cell interactions are apparently functional upon the early events of interstitial cell differentiation and function. The dramatic increase in the number of FLC during embryonic development must occur through recruitment from precursor cells because differentiated FLCs are mitotically inactive (Byskov, 1986).

The morphological characteristics of FLC have been described for both rodents and primates in several studies (reviewed in Haider, 2004) and are therefore not discussed further in this review.

Induction of FLC differentiation

One of the signalling molecules involved in FLC differentiation is hedgehog, originally discovered in body patterning of *Drosophila* larvae (Nusslein-Volhard and Wieschaus, 1980). Hedgehog signalling mediates in most tissues epithelial-mesenchymal communication. In mammals there are three hedgehog ligands: desert hedgehog (Dhh), Indian hedgehog (Ihh) and sonic hedgehog (Shh) (Varjosalo and Taipale, 2008). In the absence of ligand, the hedgehog receptors at the cell membrane, patched (Ptch1 or 2), repress another receptor, smoothened (Smo). Upon ligand binding to Ptch the repression of Smo is released and the intracellular signalling cascade is turned on, including the Gli-Krüppel family of transcription factors (Gli 1, 2 and 3).

Within the mouse embryonic testis, Dhh is expressed in embryonic day 11.5 (E11.5) Sertoli cells (Bitgood and McMahon, 1995) and Ptch1 and Gli1 in Leydig and myoid cells of the interstitium, indicating the existence of an epithelial-mesenchyme communication link (Barsoum and Yao, 2011; Franco and Yao, 2012). The early expression makes Dhh the first identified morphogenic regulator of the testis after expression of the testis-determining SRY gene. Several genetic modifications have demonstrated FLC as targets of hedgehog signalling (Park et al., 2007). Dhh knockout mice have a reduced number of FLC (Yao et al., 2002), overexpress Gli1 (Kroft et al., 2001) and show constitutive activation of Smo (Barsoum et al., 2013) resulting in FLC hyperplasia. It is possible that the downstream target gene of Dhh signalling in FLC is the orphan nuclear receptor chicken ovalbumin upstream promoter transcription factor II (Coup-tfII), as has been demonstrated in other organs (Krishnan et al., 1997; Xu et al., 2008). Another important factor is the master regulator Sfl, and with respect to FLC development and function its role in the maintenance of Sertoli cell Dhh expression and the FLC steroidogenic machinery are of particular importance (Park et al., 2005, 2007).

With regard to the role of Coup-tfII in the function of developing FLC, the existing information is somewhat conflicting. In mice with inducible knockout of Coup-tfII in late fetal life (E18.5), hypoplasia of LC in the post-natal testes is observed, indicating that this transcription factor is crucial for the maintenance and maturation of progenitor cells into mature LC (Qin et al., 2008). The induction of the knockout both prenatally and post-natally suggests that Coup-tfII is important for the maintenance and maturation of both fetal and adult progenitor LC. In contrast to these findings, van den Driesche et al. (2012) observed in mice, that when nuclear Coup-tfII expression in FLC is switched off during the course of fetal maturation, the expression of Sfl-dependent LC steroidogenic genes (steroid acute regulatory protein (StAR), P450 cholesterol side-chain cleavage (Cyp11a), P450 17 α hydroxylase/C17-20 lyase (Cyp17a)) is increased. This was interpreted to mean that COUP-TFII functions as a repressor of FLC functional maturation; when its expression is lifted, these cells acquire the steroidogenic machinery to produce testosterone for fetal masculinization. These two seemingly opposite observations on the role of Coup-tfII in FLC maturation can be reconciled by speculating that Coup-tfII maintains the pool of steroidogenically inactive precursor LC while suppressing the expression of Sfl-dependent steroidogenic genes. When its expression is switched off, by an as yet unidentified mechanism, Dhh and Sfl induce the precursor cells to mature into steroid producing LC. Hence, Dhh production by Sertoli cells, signalling through Ptch1 and Gli1 in progenitor and maturing FLCs, and subsequent down-regulation of Coup-tfII and up-regulation of the Sfl-dependent steroidogenic enzymes, form a likely regulatory cascade upon FLC maturation.

While Dhh signalling is important for the initial steps in fetal stem LC (SLC) differentiation, Notch signalling restricts the differentiation of these stem cells. Constitutive activation of Notch signalling in fetal SLC causes a dramatic reduction in FLC number and a concomitant accumulation of undifferentiated mesenchymal cells, presumptive fetal SLC, in the intersitium of the mouse testis (Tang et al., 2008). Differentiated FLC after E13.5 do not seem to be influenced by Notch signalling implicating that Notch signalling rather acts to maintain the stem cell lineage (reviewed in Svingen and Koopman, 2013).

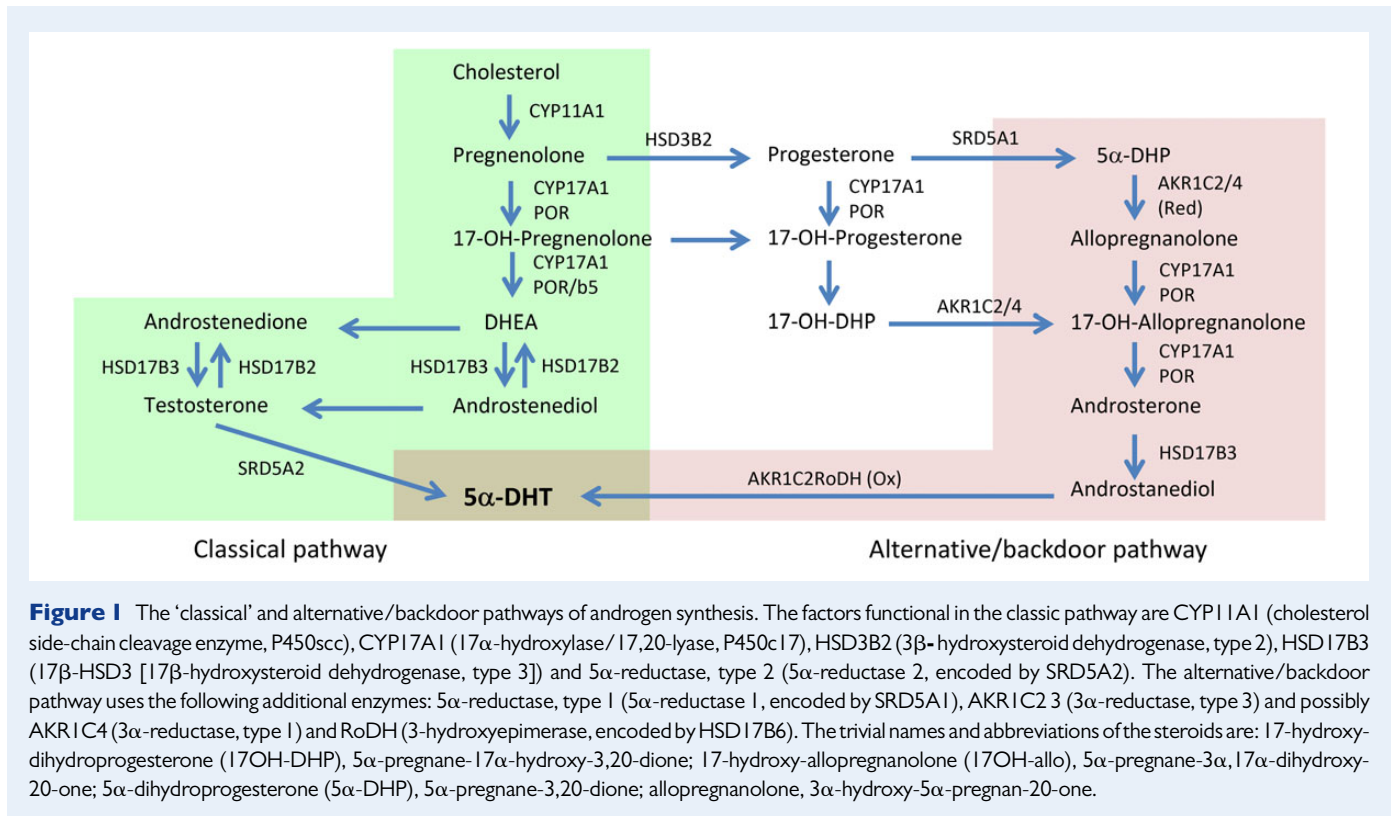
Other factors involved in fetal SLC and FLC differentiation include platelet-derived growth factor- α (Pdgfa) (Brennan et al., 2003), insulin-like growth factor I (Igf1) (Rouiller-Fabre et al., 1998; Wang and Hardy, 2004), hepatocyte growth factor (Hgf) (Ricci et al., 2012) and the homeobox gene *Aristaless* (Arx) (Kitamura et al., 2002). A recent study implicates Arx to play not only a role in stem cell differentiation but also in migration, and is thus considered to be a specific marker for fetal SLC (Miyabayashi et al., 2013). Gonadotrophins do not play a role in the formation of the FLC population in rodents as the cells acquire the expression of the LH/CG receptor (Lhcgr) (The official term for this receptor is LH/CG receptor (LHCGR). We use this term in the case of the primate receptor. Because there is no cg-b gene in the rodent genome, we use a more appropriate term, LH receptor (Lhr) for rat and mouse.) only after they have differentiated from stem cells (Majdic et al., 1998). The post-natal disappearance of FLC may be regulated by androgen-dependent production of paracrine factors in Sertoli cells (Hazra et al., 2013).

FLC function and effects of their hormone products

The main hormones produced by the mammalian fetal testes are androgens [testosterone and 5 α -dihydrotestosterone (DHT)] and InsI3 synthesized in FLC, and the Sertoli cell-derived anti-Müllerian hormone (Amh) and inhibin (Anderson et al., 2002).

Androgens. In the mouse, the expression of steroidogenic enzyme genes starts shortly after LC differentiation, on E13.0 (Greco and Payne, 1994) which is the same age when testosterone can be detected for the first time in the fetal testis (Gondos, 1980). These events occur on Weeks 6–7 of gestation in human male fetuses (Tapanainen et al., 1981; O'Shaughnessy et al., 2007). The peak testosterone level is attained around E17–18 in the mouse (Gondos, 1980; O'Shaughnessy et al., 1998) and on gestation weeks 12–14 in the human (Tapanainen et al., 1981).

In principle the production of testosterone in the fetal testis occurs in similar fashion as in the adult testis along the 'classical' steroidogenic pathways (Fig. 1), using either *de novo* synthesized cholesterol or lipoprotein-associated cholesterol from extracellular sources as substrate. Although the fetal circulation is full of suitable substrates for testosterone synthesis (especially placental progesterone), it seems that FLC steroidogenesis starts from cholesterol. There are species differences in the preference of the Δ^5 and Δ^4 pathways, the former being preferred in humans (Huhtaniemi et al., 1970; Flück et al., 2003) and the latter in mice (Greco and Payne, 1994). These differences in pathway preference are probably due to different substrate affinities of the Cyp17a C17-20 lyase reaction. The preferential use of the Δ^5 pathway by the human fetal testis may explain why placental progesterone is not a good substrate for testosterone synthesis (Huhtaniemi et al., 1970; Mathur et al., 1972). Due to the low level of steroid



sulphatase activity in fetal tissues the abundantly present adrenal dehydroepiandrosterone sulphate is also unlikely to serve as a good substrate for testosterone production.

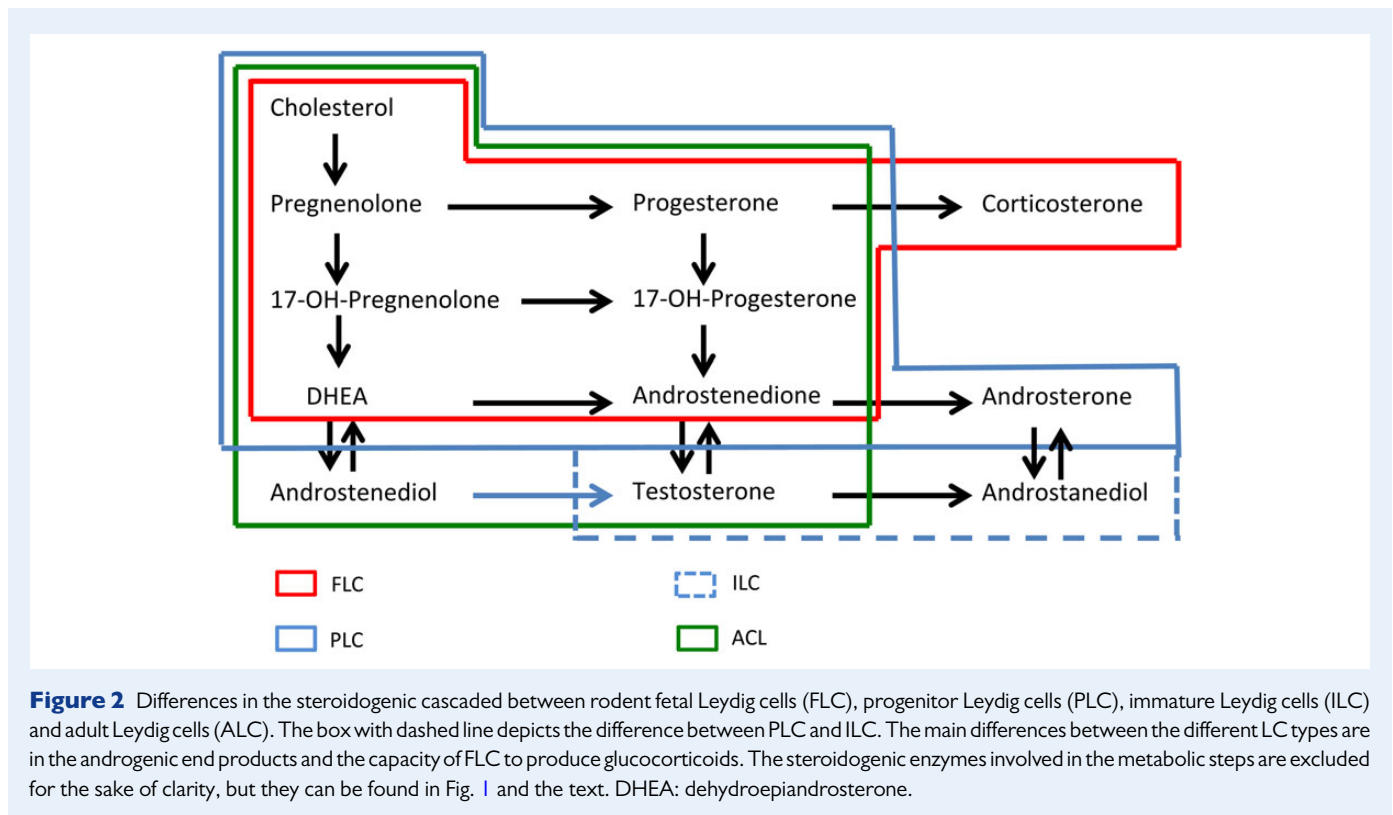
An interesting special feature of the fetal testis, at least in the mouse, is the distribution of 17 β -hydroxysteroid dehydrogenase type 3 (Hsd17b3), converting androstenedione to testosterone (O'Shaughnessy *et al.*, 2000; Shima *et al.*, 2013) (Fig. 2). In the adult testis it is confined to the interstitial LC but in the fetus to Sertoli cells of the tubular compartment, thus making fetal testosterone production dependent on the interplay of two cellular compartments. Another special feature of mouse FLC is the expression of 11 β -hydroxylase (Cyp11b1) and 21-hydroxylase (Cyp21) (Hatano *et al.*, 1996), in which sense they share functional features with adrenocortical cells. Conversely, the adrenal gland of the mouse produces androgens only in the fetal period (Keeney *et al.*, 1995).

While testosterone is the main androgen produced by the fetal testis, its conversion to the more bioactive androgen 5 α -DHT is important for masculinization of the external male genitalia. It was formerly thought that 5 α -DHT was metabolized from testosterone by the action of 5 α -reductase type 2 (SRD5A2) in genital skin. Males with inactivation of this gene have ambiguous genitalia, i.e. disorder of sexual differentiation (DSD). However, recent studies of specific cases of DSD have revealed that a significant part of fetal 5 α -DHT is produced from progesterone using the alternative or 'backdoor' metabolic pathway with 5 α -dihydroprogesterone, allopregnanolone, 17-hydroxyallopregnanolone, androsterone and androstenediol as intermediates (Fig. 1) (Flück *et al.*, 2011). The key enzymes in this pathway, not employed by the classical steroidogenic pathway, are the alfa-keto-reductases AKRC2 and AKRC4, of which the former converts androstenediol to 5 α -DHT. Because human males with SRD5A2 and AKRS2/4 deficiency present with ambiguous genitalia, both pathways are needed for normal male fetal masculinization.

The backdoor pathway of 5 α -DHT synthesis may be a general feature of mammalian fetal testes, not only confined to humans, because it has also been found in the marsupial tammar wallaby pouch young (Shaw *et al.*, 2000), fetal mouse (Mahendroo *et al.*, 2004) and rat testes (Ge *et al.*, 1999).

INSL3. The second hormonal FLC product is INSL3, which is a member of the insulin-relaxin family of peptides, acting through a G-protein coupled receptor relaxin/insulin-like family peptide receptor 2 (RXFP2) (Agoulunik, 2007). INSL3 production starts in rodent and human fetal testes around the same stage of development and appears to be largely autonomous (McKinnell *et al.*, 2005; Anand-Ivell *et al.*, 2008). The evolutionary aspects of this exclusively mammalian peptide are covered elsewhere (Ivell and Bathgate, 2007). It is expressed in both mature FLC and ALC, and its only clearly established function so far is its role, along with androgens, in the transabdominal testicular descent in fetal life. Its expression in the mouse fetal testis has been detected as early as on E12.5 (Sarraj *et al.*, 2010), before the onset of the transabdominal phase of testicular descent (Zimmermann *et al.*, 1997). In humans, INSL3 has been detected in amniotic fluid in Week 11 of gestation (Anand-Ivell and Ivell, 2014). INSL3 gene expression is regulated by multiple *SFI* response elements in its promoter region (Sadeghian *et al.*, 2005), and the level of Insl3 expression in the fetal rodent testis follows in a constitutive fashion the FLC maturational stage and LH responsiveness, though independently thereof (Balvers *et al.*, 1998; Bay and Anand-Ivell, 2014). Likewise, INSL3 levels in humans show a peak during the post-natal 'mini-puberty', as a sign of transient LC activation (Bay *et al.*, 2007).

The role of Insl3 and its cognate receptor Rxfp2 in testicular descent has been proven by knockout mice for the cognate gene, presenting with



high intra-abdominal cryptorchidism (Zimmermann *et al.*, 1997; Nef and Parada, 1999; Gorlov *et al.*, 2002; Overbeek *et al.*, 2011). Rxfp2 is expressed in multiple cell types of the male and female urogenital organs, but its functionally significant expression is confined to the gubernaculum (Ivell and Bathgate, 2007). In line with this *Insl3* is able to stimulate the growth of gubernaculum cells *in vitro* (Kumagai *et al.*, 2002), which *in vivo* leads to thickening of the gubernaculum with concomitant traction of the testes to the lower part of the abdominal cavity. Interestingly, female transgenic mice overexpressing *Insl3* present with transabdominally descended ovaries (Adham *et al.*, 2002; Koskimies *et al.*, 2003).

Normal and disturbed regulation of FLC

Gonadotrophins. It is textbook knowledge that FLC androgen production induces masculinization of the extra-gonadal genitalia of a male fetus, and that 5α -DHT is needed for the masculinization of external genitalia. The old findings on endogenous steroid levels in the human fetal testis (Huhtaniemi *et al.*, 1970; Tapanainen *et al.*, 1981) and their responsiveness to hCG stimulation *in vitro* (Huhtaniemi *et al.*, 1977) have been confirmed and extended by more recent studies at the genetic level (O'Shaughnessy *et al.*, 2007). Expression of the *LHCGR* mRNA in human fetal testis is detectable at least in Week 11 of gestation (O'Shaughnessy *et al.*, 2007; Fowler *et al.*, 2009), most likely even earlier, and the concentration of hCG in fetal circulation during the peak of testosterone secretion on Weeks 12–14 is over 10-fold higher than that of LH (Fowler *et al.*, 2009). The decline in testosterone levels during the second trimester shows good correlation with that of hCG but not of LH. Hence, FLC responsiveness to placental hCG stimulation, rather than pituitary LH, suggests that hCG provides the main tropic stimulus at this stage of FLC development.

Further evidence for the importance of hCG and LHCGR in the regulation of human FLC steroidogenesis is provided by inactivating mutations of *LHCGR* and *LHB* genes (Themmen and Huhtaniemi, 2000). Genetic males with a *LHCGR* mutation lack normal intrauterine masculinization of genitalia and present with the phenotype of pseudo-hermaphroditism (Themmen and Huhtaniemi, 2000). In contrast, those with an *LHB* mutation are normally masculinized at birth, because hCG provides sufficient tropic stimulation for FLC (Valdes-Socin *et al.*, 2004; Lofrano-Porto *et al.*, 2007) (Fig. 3). The absence of LH becomes evident only at puberty when ALC maturation and androgen production do not occur. There is some controversy whether FLC androgen production in humans is initiated independent of gonadotrophic hormone stimulation (reviewed in Scott *et al.*, 2009). A study reporting a case of pseudo-hermaphroditism in males due to a homozygous missense mutation of the *LHCGR* gene suggests that human FLC steroidogenesis may begin independent of tropic hormone stimulation (Kremer *et al.*, 1995), as is the case in rodents (see below).

The situation in rodents is somewhat different from primates, one reason being that there is no choriongonadotrophin in these species. Several pieces of evidence show that in rodents, FLC steroidogenesis starts autonomously (or at least independent of LH stimulation). In the mouse, the FLC start producing testosterone (E13.0) (Gondos, 1980) before the onset of pituitary *Lhb* (E16.0) expression (Japon *et al.*, 1994) and testicular expression of full-length *Lhr* mRNA (E16.0), capable of encoding the functional receptor protein (O'Shaughnessy *et al.*, 1998). Similar findings on earlier expression of steroidogenic enzymes than of *Lhr* have been made in fetal rat testes (Gangnerau and Picon, 1987; Zhang *et al.*, 1994; Majdic *et al.*, 1998). However, although the peak of mouse FLC testosterone production at E18.0 parallels the peaks of serum LH levels and testicular *Lhr* expression (O'Shaughnessy *et al.*,

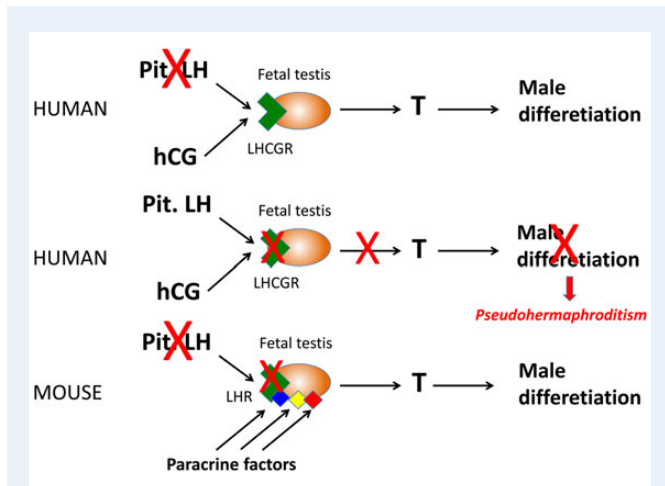


Figure 3 The difference in regulation of FLC testosterone production in the human and mouse. *Upper panel:* In principle two tropic hormones, pituitary LH and placental hCG can stimulate FLC LH/CG receptor (LHCGR) (Lhr) and thereby activate androgen biosynthesis. Pituitary LH is dispensable, because if it is eliminated through mutation, FLC steroidogenesis is unaffected. Inactivating mutations in hCG genes are not known because apparently they would inhibit pregnancy. Inactivating Lhr mutations cause pseudohermaphrodite phenotype in genetic males (i.e. lack of intrauterine masculinization due to defective FLC testosterone (T) production). *Lower panel:* In the mouse, FLC androgen production is possible in the absence of pituitary LH or LHR, as demonstrated by knockout mouse models, and in these cases a plethora of paracrine factors are able to induce FLC testosterone production and intrauterine masculinization.

1998), this does not mean that rodent FLC steroidogenesis is LH dependent. Hypogonadotrophic mice due to a spontaneous inactivating GnRH mutation (hpg mice) (Cattanach *et al.*, 1977), or gonadotrophin common α -subunit (Kendall *et al.*, 1995), Lhb (Ma *et al.*, 2004) or Lhr (Zhang *et al.*, 2001) knockout mice, are normally masculinized at birth despite complete absence of LH stimulation of the FLC *in utero*. This is in contrast to humans, as males with an inactivating mutation of the LHCGR present with severely underdeveloped external genitalia (reviewed in Svechnikov *et al.*, 2010). Hence, the initiation of FLC steroidogenesis in humans, like in rodents, may be gonadotrophin independent, but before the end of the first trimester of pregnancy gonadotrophins become indispensable for steroid production and normal masculinization.

Additional evidence for gonadotrophin independence is provided by knockout mice for the thyroid-specific enhancer-binding protein (T/ebp) transcription factor (also called NK2 homeobox 1 (NKX2.1) or thyroid transcription factor 1), known to control organogenesis and transcription in the thyroid gland, lung, ventral forebrain, and pituitary gland (Pakarinen *et al.*, 2002). These mice, despite the absence of a functioning pituitary gland, still show normal masculinization at birth even though their intratesticular testosterone levels are only 5–10% of the control concentrations. Another fact this experiment demonstrates is that there is a large safety margin in the concentration of testosterone needed to stimulate fetal masculinization, because it can occur at testosterone levels < 10% of normal. Hence, we can conclude that rodent FLC steroidogenesis begins independent of LH stimulation, attains subsequently LH-responsiveness, but does not become LH dependent until after birth. The recent knockout of the Kiss1 gene provides further

evidence for the independence of the regulation of rodent fetal testicular function on hypothalamic-pituitary level (Poling and Kauffman, 2012); despite reduced gonadotrophin levels, neonatal Kiss1 knockout male mice have unaffected testosterone production and are normally masculinized.

Paracrine regulation. Because of the independence of rodent FLC on LH stimulation, there must be other factors responsible for the stimulation of their steroidogenesis. In fact, FLC are found to be in this respect rather promiscuous, and several factors, both endocrine and paracrine, have been identified to stimulate specifically FLC, but not ALC (Fig. 3). Such factors include at least the hypothalamic corticotrophin releasing hormone (CRH) (McDowell *et al.*, 2012), pituitary adrenocorticotrophic hormone (ACTH) (O'Shaughnessy *et al.*, 2003), and local para/auto-crine factors such as Dhh, Pdgfa, Igf1, vasoactive intestinal peptide, pituitary adenylate cyclase-activating polypeptide, natriuretic peptides, hepatocyte growth factor and the transforming growth factor (TGF)- β family peptides (El-Gehani *et al.*, 1998, 2000, 2001; Griswold and Behringer, 2009; Scott *et al.*, 2009; Sarraj *et al.*, 2010; Ricci *et al.*, 2012). Which of these factors are of critical importance remains to be studied. For example, pro-opiomelanocortin (Pomc) knockout mice lacking ACTH and Lhr/Pomc combined knockout mice are normally masculinized, indicating that neither ACTH nor LH action is essential for FLC steroidogenesis (O'Shaughnessy *et al.*, 2009). Neither is there evidence for the lack of masculinization in mice devoid of thyroid-stimulating hormone, growth hormone or prolactin (Ormandy *et al.*, 1997; Zhou *et al.*, 1997; Marians *et al.*, 2002). There is apparently a fair amount of redundancy in the stimulation of FLC, to ensure the occurrence of such an important maturational step as masculinization.

Much less is known about the role of these paracrine factors in human FLC development and function. PDGFA, PDGFB and their receptors as well as the transcription factor GATA-4 have been demonstrated to be highly expressed in FLC between Weeks 14 and 20 of gestation when testosterone levels are high (Ketola *et al.*, 2000; Basciani *et al.*, 2002; reviewed in Svechnikov *et al.*, 2010). It seems however unlikely that these factors stimulate FLC function as in the absence of functional LH/hCG normal masculinization is inhibited in human males (Themmen and Huhtaniemi, 2000). All in all, it is curious that the masculinization of male fetuses through FLC testosterone production in men and mice seem to employ such different backup mechanisms, i.e. hCG versus paracrine factors.

FLCs as target of endocrine disruptors

The function of FLCs, including in humans, has recently received increased attention because of the fears of these cells being a sensitive target for demasculinizing effects of endocrine disrupting chemicals (EDCs) through interference with their androgen production [see, e.g. (Svechnikov *et al.*, 2010; Albert and Jegou, 2013; Martinez-Arguelles *et al.*, 2013)]. While there is plethora of studies *in vivo* and *in vitro* on anti-androgenic and/or estrogenic inhibitory effects of EDCs (mostly phthalates and bisphenol A) on FLCs in various animal models, there are large species differences in the actions of these chemicals. The findings of EDC effects on rat, mouse and human FLC testosterone and INSL3 production are rather conflicting, and it is difficult to decipher the salient phenomena from the existing data (Johnson *et al.*, 2012; N'Tumba-Byn *et al.*, 2012; Moody *et al.*, 2013). Cultured human fetal testes (Hallmark *et al.*, 2007) and the technically elegant human fetal testicular xenograft studies

in nude mice have reported negative and/or inconclusive findings on deleterious effects of EDCs (Heger *et al.*, 2012; Mitchell *et al.*, 2012; Spade *et al.*, 2014). Somewhat counterintuitive findings were made in a recent study reporting stimulatory effects of the analgesics aspirin and indomethacin, at doses relevant to human exposure, on testosterone production in human fetal testicular cultures, while N-(4-hydroxyphenyl)-arachidonylethanolamide (AM404) and paracetamol (acetaminophen) decreased INLS3 production (Mazaud-Guittot *et al.*, 2013). Similar species differences, with human FLC being resistant to deleterious effects, have been reported with the estrogenic compound diethylstilbestrol (Mitchell *et al.*, 2013). An interesting finding is that maternal smoking reduces DHH expression during fetal testicular development, which might explain the (in some cases) impaired reproductive development of offspring from such pregnancies (Fowler *et al.*, 2008).

Besides the conflicting results between various animal models, another caveat in the field of EDC research is whether positive findings have been made using doses that have relevance for real-life exposure from the environment. The field is currently polarized between proponents and sceptics with respect to the importance of health hazards of EDCs. An interesting conclusion of a review on EDCs (vom Saal and Hughes, 2005) is that the harmful effects are likely to be found in research carried out with governmental funding, whereas no harm is found when the funding comes from industrial sources! Better models to monitor human fetal testicular function, and in particular FLC function, continue undoubtedly to be on the agenda of future EDC research.

MAMLD1. Besides cryptorchidism, hypospadias is another common disturbance in the development of male genitalia. Mutations and polymorphisms have been detected in the *Mastermind Containing 1* (*MAMLD1*) gene on human chromosome Xq28 in hypospadias patients, with and without associated genital abnormalities, such as micro-penis and cryptorchidism (Ogata *et al.*, 2012). A recent *Mamld1* knockout mouse model (Miyado *et al.*, 2012) shows that this gene specifically augments FLC gene expression, and although the mice do not have a significant reproductive phenotype the borderline suppression of FLC function may explain the reproductive phenotypes in affected humans. It is apparent that additional genes involved in the function of FLC will be identified in the future.

An emerging topic is the role of microRNAs in the regulation of testicular differentiation and function (Rakoczy *et al.*, 2013).

ALC

Cellular origin of ALC

There is no doubt that like the FLC population, ALC arise from stem cells, which have their origin in the fetal testis. However, to date, it remains unclear whether these distinct cell populations share a common stem cell ontogeny. Support for a common ontogeny of both cell populations comes from similarities in the regulation of their development (Ge and Hardy, 2007) as well as the presence of common markers. For instance Dhh and Pdgfra not only stimulate the differentiation of stem cells into testosterone producing FLC (Yao *et al.*, 2002; Brennan *et al.*, 2003), but also play a role in ALC development, as gene deletions suppressing Dhh and Pdgfra expression influence the formation of the ALC population (Clark *et al.*, 2000; Gnessi *et al.*, 2000). COUPTF-II, initially considered a marker for fetal SLC in rats and mice (van den Driesche *et al.*, 2012), has recently been identified in SLC that give rise to the ALC population

(Kilcoyne *et al.*, 2014). Another piece of evidence comes from a study by Park *et al.* (2007) who showed that Sfl haploinsufficiency in combination with ablation of Dhh gene expression led to a complete inhibition of the formation of both FLC and ALC populations. In support of this observation, increased activation of the Dhh pathway in the SLC population during fetal life alters the dynamics of this population and consequently the numbers of ALC in adulthood (Barsoum *et al.*, 2013).

Other lines of evidence however contradict a common stem cell ontogeny for FLC and ALC, pointing to functional differences between the two LC origins. For instance mature ALC development and androgen production are highly dependent on the presence of LH and functional Lhr, while FLC development and steroidogenesis are responsive to, but not dependent on LH action, as seen in LH receptor knockout (LuRKO) mice (Zhang *et al.*, 2001, 2004). Another example is the observation that FLC in the mouse testis respond to ACTH (O'Shaughnessy *et al.*, 2003), and rat FLC to a vast number of regulatory peptides (see above), with pronounced increase in testosterone production, but these compounds have no effect on ALC androgen production. Hence, to answer the question as to whether FLC and ALC share a common ontogeny, more information is needed concerning the cellular characteristics and pattern of gene expression, as well as on lineage tracing studies, in both FLC and ALC stem cells.

Induction of ALC differentiation

The formation of the ALC population along the LC lineage can be divided into four steps, based on developmental changes in cell morphology and steroidogenic capacity. The next section of this review will describe this process with specific emphasis on the relation between cell function and morphology.

From stem cell to progenitor cell. The post-natal development of the ALC population traces back to the stem cell stage. These SLC, which have an indifferent mesenchymal-like morphological appearance and do not express lineage specific markers such as Lhr and steroidogenic enzymes, were identified and isolated several years ago (Ge *et al.*, 2006). These cells are further characterized, like FLC, by the expression of Pdgf receptor a (Pdgfra), Gata4, and the stem cell markers leukaemia inhibiting factor receptor (Lifr) and c-kit. The proliferative activity of these stem cells is directly stimulated when Lif, stem cell factor (Scf), epidermal growth factor (Egf) and Pdgfa are added to the culture medium. Differentiation of these stem cells to Lhr expressing, steroid producing progenitor LC (PLC, the second stage of ALC development) can be induced by culturing the cells in the presence of LH, IGF-I and the active thyroid hormone tri-iodothyronine (T3), or by transplanting the cells immediately after isolation into a testis depleted of LC (Fig. 4) (Ge *et al.*, 2006). Further support for a role of IGF-I and T3 in SLC differentiation comes from *in vivo* studies. In mice with a targeted deletion of the Igf1 gene, the differentiation of SLC into PLC is delayed, though not completely inhibited (Wang and Hardy, 2004; Hu *et al.*, 2010).

While daily neonatal T3 treatment of new-born rat pups advances the differentiation of SLC into PLC (Teerds *et al.*, 1998), suppression of T3 levels has the opposite effect. Initially it was even thought that neonatally induced hypothyroidism by treatment with the goitrogen 6-propyl-2-thiouracil (PTU) completely prevents the differentiation of SLC into PLC (Mendis-Handagama *et al.*, 1998; reviewed in Ariyaratne *et al.*, 2000a). More recent studies have shown that under PTU or dietary-induced hypothyroid conditions the differentiation of SLC into

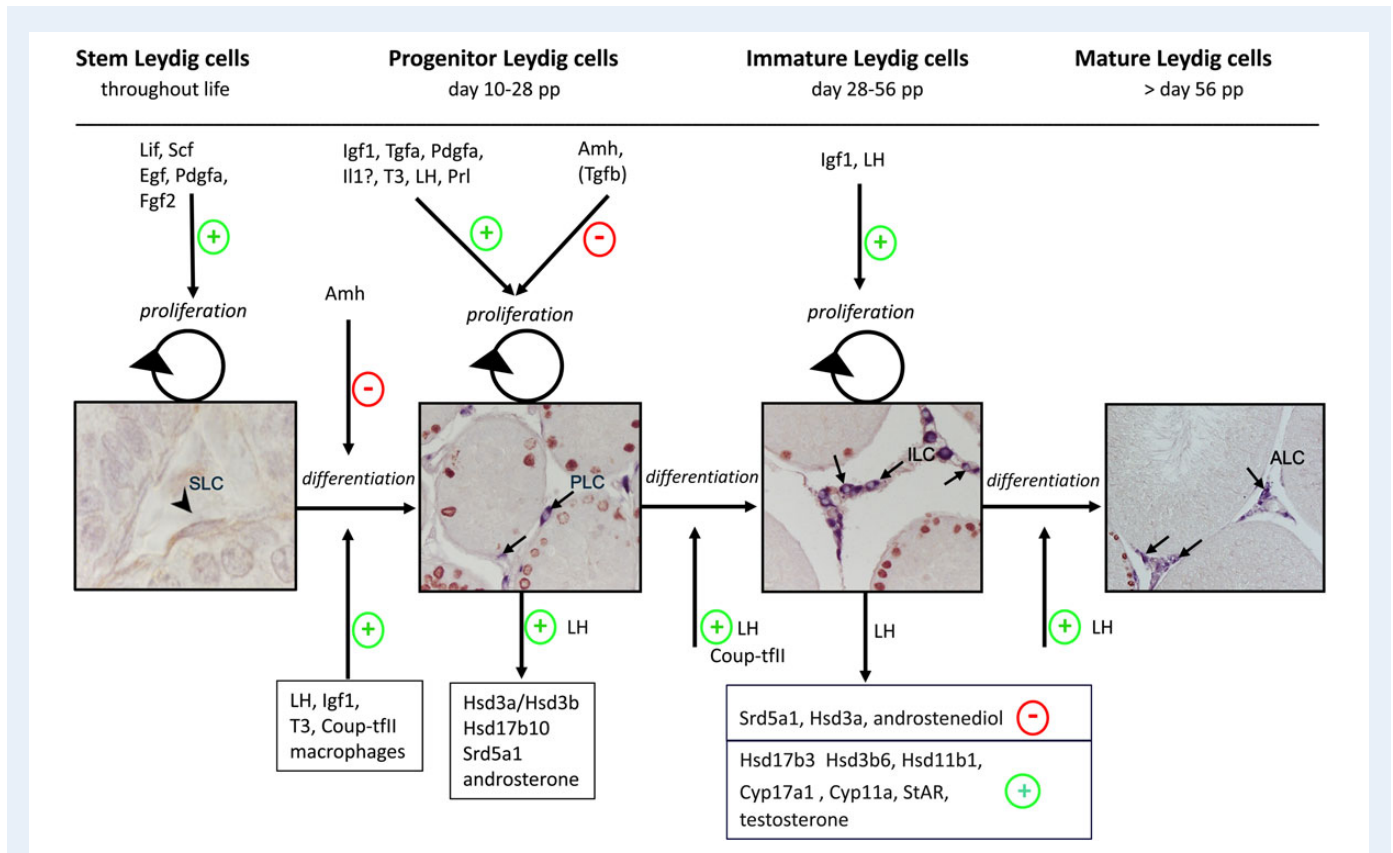


Figure 4 LC development in the rat testis. Stem Leydig cells (SLC) are characterized by their spindle-shaped morphology and the presence of platelet-derived growth factor receptor A (Pdgfra) as depicted by the brown cytoplasmic staining (testis section of a 12-day-old rat testis was stained with an antibody against Pdgfra, magnification $\times 750$). Under the influence of different factors these SLC proliferate and differentiate into PLC which express Lhr and 3β-hydroxy steroid dehydrogenase (Hsd3b) (section of a 21-day-old rat testis stained with antibodies against the proliferation marker 5-bromo-2-deoxyuridine (BrdU, brown nuclear staining) and the steroidogenic enzyme Hsd3b (blue cytoplasmic staining), magnification $\times 360$). PLC undergo several cycles of cell proliferation. This process is regulated by both stimulatory and inhibitory factors (indicated by + and -). The major androgens produced by PLC are metabolites of testosterone. Around the age of 28 days, under the influence of LH PLC differentiate into ILC (section of a 35-day-old rat testis stained with antibodies against BrdU (brown nuclear staining) and Hsd3b (blue cytoplasmic staining), magnification $\times 290$). ILC undergo one more cycle of proliferation after which they continue to differentiate. During their differentiation into mature Leydig cells a switch in steroid production occurs. The activity of Srd5a1 and 3α-hydroxysteroid dehydrogenase (Hsd3a) drops, while the activity of other steroidogenic enzymes, among which is Hsd17b3, increases making testosterone now the major androgen produced by LC. Around the age of 56 days post-partum most ILC have differentiated into mature ALC. The ALC population is a stable population of cells with an extremely low proliferative capacity, but also a dynamic population of cells with a significant regenerative capacity (section of a 70-day-old rat testis; ALC are stained with an antibody against Hsd3b (blue cytoplasm), magnification $\times 110$). Lif: leukaemia inhibiting factor; Scf: stem cell factor; Egf: epidermal growth factor; Pdgfa: platelet-derived growth factor A; Fgf2: fibroblast-like growth factor type 2; Amh: anti-Müllerian hormone (Amh); Igf1: insulin-like growth factor I; Tgfa: transforming growth factor α; Il1: interleukin 1; T3: triiodothyronine; Prl: prolactin; Tgfb: transforming growth factor β; Coup-tfll: chicken ovalbumin upstream promoter transcription factor II; Hsd17b10: 17β-hydroxysteroid dehydrogenase type 10; Hsd3b6: 3β-hydroxysteroid dehydrogenase type 6; Hsd11b1: type 11β-hydroxysteroid dehydrogenase; StAR: steroid acute regulatory protein.

PLC is not inhibited but delayed (Rijntjes *et al.*, 2009). This seems in contrast to the observations made by Ge and colleagues in their *in vitro* studies (Ge *et al.*, 2006). However, one has to keep in mind that under *in vivo* conditions there is still some T3 present in the circulation and thus in the testis. This may be enough to facilitate the transition from SLC into PLC, though at a lower rate, stressing the indispensable role of T3 in this developmental process.

Besides T3, LH appears to play a role of the utmost importance in this developmental process as in LuRKO mice ALC development is completely inhibited (Zhang *et al.*, 2001, 2004; Lei *et al.*, 2004). *In vitro* experiments have confirmed these *in vivo* observations (Teerds *et al.*,

2007), emphasizing the important difference between FLC and ALC development, as reviewed above.

Differentiation of SLC into PLC is a balanced process, which is not only dependent on stimulatory but also on inhibitory factors. Sertoli cell derived Amh is such an inhibitory factor, as Amh overexpression leads to accumulation of mesenchymal SLC in the interstitium and a reduced number of immature LC (ILC) and mature ALC. In accordance with these observations, in the absence of Amh this differentiation process is enhanced (Lyet *et al.*, 1995; Racine *et al.*, 1998).

Other factors and cells have been implicated in the process of LC differentiation as well. An example of a transcription factor that is involved in ALC

development is Coup-tfll. Time-specific ablation of Coup-tfll during late embryonic development results in a delay in PLC formation, similar to the response to Amh overexpression. These observations suggest that Coup-tfll may be required for LC lineage commitment (Qin *et al.*, 2008).

A cell type suggested to be important for ALC development is the testicular macrophage. *In vivo* studies by Gaytan *et al.* (1994) have shown that in the absence of macrophages differentiation of SLC into PLC is completely inhibited. These observations are however puzzling as Ge *et al.* (2006) demonstrated that it is possible to induce SLC differentiate in PLC *in vitro* following the addition of T3, LH and IGF-I. None of these factors are specifically macrophage derived.

Based on the above observations we can conclude that the past years have contributed significantly to our understanding of the first developmental steps of the LC lineage (Fig. 4). Notwithstanding, several questions still remain unanswered such as the role of macrophages in this process.

From progenitor cell to ILC. Depending on the strain of rats, the first PLC develop between Days 10 and 13 post-partum. In general these cells are located in close vicinity to the seminiferous tubules, although some reports indicate that they can also be found in the perivascular region. PLC are identified by their spindle-shaped morphology, the presence of a limited amount of cytoplasmic organelles involved in steroid production, such as smooth endoplasmic reticulum (SER), tubulovesicular mitochondria and lipid droplets, and the presence of Lhr, androgen receptor, Hsd3b and 17 β -HSD (Hsd17b) activity (reviewed in Haider, 2004).

Although PLC are capable of producing androgens, the expression pattern of some of the steroidogenic enzymes differs from mature ALC (Fig. 2). Like ALC, PLC express StAR, Cyp11a1 and Cyp17a1 although at somewhat lower levels (Kanzaki and Morris, 1999; Dong *et al.*, 2007). In contrast to ALC, the expression of 3 β -HSD type 6 (Hsd3b6) is low (Baker *et al.*, 1999), the expression of 17 β -HSD type 3 (Hsd17b3) is negligible (Dong *et al.*, 2007) while 17 β -HSD type 10 (Hsd17b10) is first detected in PLC around Day 20 post-partum (Ivell *et al.*, 2003). Type I 11 β -HSD (HSD11b1) activity is low, and at this stage of LC development HSD11b reductive activity predominates over oxidative activity. Hsd11b1 is postulated to increase intracellular concentrations of active glucocorticoids, which can potentiate the sensitivity of PLC to LH and stimulate the expression of Srd5a1 and Hsd3a (Ge *et al.*, 1997). This transient developmental rise in the expression levels of Srd5a1 and Hsd3a together with the low expression levels of Hsd3b6 and Hsd17b3 make androsterone and androstenedione the major steroids produced by PLC (Viger and Robaire, 1995; Dong *et al.*, 2007; Wu *et al.*, 2007; Figs 2 and 4).

Pulse-chase experiments with [³H]-thymidine show that PLC undergo several cycles of proliferation before they differentiate into ILC (Hardy *et al.*, 1989). Support for these initial observations comes from later studies on the expression of genes that are markers for cellular proliferation. PcnA, cyclin A2 and D3 expression are highest in PLC compared with later stages of LC development (Ge and Hardy, 1997; Sriraman *et al.*, 2000). A gene cluster analysis performed on highly purified PLC showing high expression of genes involved in cell cycle regulation, e.g. Cdc25b, Cdc20 and genes encoding growth factor receptors, e.g. Pdgfra, and interleukin-6-receptor beta chain (Il6st), extends these observations. The expression levels of these genes decrease when PLC undergo transition into ILC (Ge *et al.*, 2005a).

Several studies have focused on the role of specific locally produced growth factors in the regulation of PLC proliferation, offering further support to the study by Ge *et al.* (2005a). Growth factors like Pdgfa, transforming growth factor α (Tgfa) and Igf1 are locally produced in the (pre)pubertal testis at the time of PLC proliferation. Knockout studies have demonstrated that the Sertoli cell derived growth factor Pdgfa is a potent stimulator of PLC proliferation. Gnessi *et al.* (2000) showed that PLC proliferation in Pdgfa knockout mice is negligible at Day 18 post-partum, an age at which PLC show significant proliferative activity in the wild type (wt) mice.

The presence of Tgfa mRNA has been demonstrated in Sertoli cells, peritubular/myoid cells and PLC (Skinner *et al.*, 1989; Mullaney and Skinner, 1992), while Cailleau *et al.* (1990) and Handelsman *et al.* (1985) reported Igf1 production and release by Sertoli cells and peritubular/myoid cells. PLC as well as more mature LC possess both Egfr receptors to which Tgfa can bind (Suarez-Quian *et al.*, 1989), and Igf1 receptors (Handelsman *et al.*, 1985), implying that Sertoli cell and peritubular/myoid cell derived Tgfa and Igf1 may play a role in the paracrine regulation of PLC proliferation *in vivo*. Support for this assumption comes from *in vitro* studies, in which it is shown that these two growth factors can stimulate PLC DNA synthesis. When a low dose of LH is added concomitantly to these cell cultures, a synergistic effect is observed (Khan *et al.*, 1992a). Further evidence for a role of Igf1 in the formation of ALC comes from *in vivo* studies. Deletion of the Igf1 gene in mice causes a reduction in the bromodeoxyuridine labelling index of PLC from post-natal day 14 through 35, leading to a reduced number of LC in adulthood (Hu *et al.*, 2010).

Another growth factor that has a stimulatory effect on PLC proliferation *in vitro* is TGF- β , although its effects are small in comparison to those of IGF-I and TGF- α . However, when added together with TGF- α and IGF-I, TGF- β appears to significantly attenuate PLC DNA synthesis (Khan *et al.*, 1992a). *In vivo* studies have confirmed the production and presence of Tgfb during testicular development. Tgfb1, the most prominent type of TGF- β present in the testis, is produced by Sertoli cells (Skinner and Moses, 1989) and detected immunohistochemically in PLC. Around Day 21-post-partum, ~50% of the PLC stain positively for Tgfb1; thereafter their proportion decreases rapidly to become undetectable in LC by the age of 35 days (Teerds and Dorrington, 1993). The pattern of Tgfa staining is opposite to Tgfb1 staining; the proportion of Tgfa positive PLC rapidly increases from Day 21-post-partum onwards (Teerds *et al.*, 1990). Considering the physiological relevance of these observations, it seems likely that the role of Tgfb in the regulation of PLC proliferation is probably minor and directed to the initial phase of PLC proliferation (Fig. 4). As TGF- β is also a potent inhibitor of PLC androgen synthesis, it has been postulated that the major function of Tgfb in PLC development is to keep androgen production in abeyance (Khan *et al.*, 1999).

In vitro studies have further demonstrated that PLC DNA synthesis can be enhanced by interleukin-1 β (IL-1 β) (Khan *et al.*, 1992b). III is locally produced by testicular macrophages, underscribing a role for this cell type in the formation of the adult LC population (Gaytan *et al.*, 1994; Cuducini *et al.*, 1997). Despite this, the role of IL-1 in this phase of testicular development has been critically questioned. Although III receptors have been localized in the interstitial compartment (Takao *et al.*, 1990), LC development and function, as well as spermatogenesis, are not affected in III type I receptor knockout mice in the absence of III receptor signalling (Cohen and Pollard, 1998). Hence, although IL-1 β has a

significant stimulatory effect on PLC proliferation *in vitro*, its role *in vivo* seems to be easily overtaken by other factors.

Next to affecting the transition of SLC into PLC, AMH plays a role in later stages of LC development as deletion of the *Amh* gene leads to LC hyperplasia in adulthood, suggestive of a role in LC proliferation (Behringer *et al.*, 1994; Wu *et al.*, 2005). Whether AMH is only involved in the regulation of PLC proliferation or also influences ILC proliferation is less obvious because there is only indirect evidence available. Salva *et al.* (2004) used the ethane dimethyl sulfonate (EDS) treated adult rat model to investigate the role of AMH in PLC proliferation. EDS is a cytotoxic drug that specifically destroys LC in the adult rat testis, a process followed by complete regeneration of the LC population to its original size. This regeneration process has many similarities with the formation of the ALC population in the post-natal rat testis (reviewed in Teerds and Rijntjes, 2007). By depleting the testis of ALC and concomitant intratesticular injection of AMH Salva *et al.* (2004) showed that AMH not only inhibited the transition of SLC into PLC but also attenuated PLC proliferation. Based on the similarities in ALC development in the post-natal testis and after EDS administration, we postulate that in the prepubertal rat *Amh* also negatively regulates PLC proliferation (Fig. 4).

AMH also influences the steroidogenic capacity of LC. Racine *et al.* (1998) elegantly showed that plasma testosterone levels are significantly reduced in mice overexpressing *Amh*. Using highly purified LC preparations the same group showed that this reduction was due to a dramatic decrease in *Cyp17a1* mRNA expression. Besides *Hsd3b* and *Cyp11a1* mRNA levels are significantly lower compared with the controls. Intratesticular AMH injection confirmed these results (Sriraman *et al.*, 2001). In a follow-up to these studies, Wu *et al.* (2005) postulated that *Amh* ablation also affects LC androgen production *in vivo*, as a more prepubertal pattern of androgen release is observed in 36-day-old mice. These observations are suggestive of a delay in ILC development in *Amh* knockout mice. In adulthood, despite LC hyperplasia, androgen production in the knockout mice is not different from wild type control animals. The latter is explained by a 42% reduction in the capacity per LC to produce testosterone (Wu *et al.*, 2005). Hence, both *Amh* overexpression as well as ablation negatively influences LC androgen production in adulthood.

The transcription factor Coup-tfII plays a role in the transition of SLC into PLC and promotes the maturation of PLC into ILC. Ablation of Coup-tfII at Day 14-post-partum does not influence the formation of PLC, however, maturation of these cells into ILC appears to be arrested in adult mutant mice. Expression of the steroidogenic enzymes *Cyp11a1*, *Hsd3b* and *Cyp17a1* is significantly lower compared with the age-matched controls, while expression of the LC differentiation marker *Ins3* is even 90% reduced. Together these observations suggest that Coup-tfII is of great importance for the process of LC differentiation, in particular the step involving the transition of PLC into ILC (Qin *et al.*, 2008).

Besides locally produced factors and transcription factors, some circulating hormones have been implicated to play a role in the process of PLC proliferation and differentiation. An important circulating hormone is LH, which is not only essential for the transition of SLC into PLC but also stimulates PLC proliferation. Guo *et al.* (2012) reported that LH maintained the proliferative capacity of PLC at 73% of control levels in the testes of rats treated with a GnRH antagonist.

Prolactin seems a hormone that may also be involved in these processes. PLC express prolactin receptors and experiments in

hypophysectomised prepubertal rats have shown that prolactin stimulates their proliferation (Dombrowicz *et al.*, 1992). Whether LH and PRL directly or indirectly, via autocrine interaction, affect PLC proliferation and functioning is not clear at present.

Thyroid hormone is another example of a peripherally produced hormone essential both for the transition of SLC into PLC and PLC proliferation. Daily neonatal T3 injections both advance the transition of SLC into PLC and stimulate PLC DNA synthesis, while reduced thyroid hormone levels delay this developmental process (Teerds *et al.*, 1998; Rijntjes *et al.*, 2009).

Taken together, these observations show that a significant number of factors have now been identified as playing roles in PLC proliferation and differentiation (summarized in Fig. 4). The role of the nuclear orphan receptor Coup-tfII in this process (Qin *et al.*, 2008) suggests that in identifying important players in the different steps along the LC lineage we should not only focus on growth factors and hormones but also take into account transcription factors that have been identified to be important for stem cell and progenitor cell differentiation.

From ILC to mature ALC. With the differentiation of PLC into ILC the cellular characteristics and position of the cells in the interstitium changes. In the rat model around Day 28 post-partum, more and more LC translocate to the centre of the interstitium. At the same time the shape of the cells is changing from spindle-shaped to oval. The nuclei of the cells become large and round with condensed heterochromatin; the cytoplasm contains now a well-developed Golgi apparatus, considerable amounts of tubulovesicular mitochondria and numerous small lipid droplets. The cholesterol stored in these lipid droplets serves as substrate for steroid production. Based on these morphological characteristics and specific functional qualities these cells are considered to be ILC. The differentiation and function of ILC is highly dependent on LH (reviewed in Haider, 2004).

With the formation of ILC the expression levels of the different steroidogenic enzymes start to change. *Hsd11b1* reductive activity increases despite *Hsd3a* activity initially being still relatively high (Ge *et al.*, 1997). After the age of 28 days *Srd5a1* activity decreases slowly; by Day 91 post-partum, when all ILC have differentiated into ALC, *Srd5a1* activity has become negligible (Viger and Robaire, 1995; Ge and Hardy, 1998).

With the differentiation of PLC into ILC the level of *Hsd3b6* and *Hsd17b3* start to increase. Due to the initially still high levels of *Hsd3a* activity the major steroid produced by young ILC is androstanediol (Fig. 2). As the ILC mature along the LC lineage the expression levels of *Hsd3b6* and *Hsd17b3*, as well as that of *StAR* and *Cyp11a1*, further increase, resulting in a gradual shift from androstanediol to testosterone production (Ge and Hardy, 1998; O'Shaughnessy *et al.*, 2000) (Fig. 2).

In contrast to PLC, ILC have only a limited proliferative capacity. Hardy *et al.* (1989) calculated that after their transition into ILC the cells undergo only one more cycle of proliferation. The mRNA levels of the proliferation markers *Pcna* and *Cyclin D3* are significantly lower in ILC compared with PLC (Sriraman *et al.*, 2000). The same holds true for mRNA and protein levels of *Cyclin A2*, while the level of the cell cycle inhibitor *Cyclin G1* at the same time increases (Ge and Hardy, 1997). These observations are further extended by a gene cluster analysis, confirming a significant decrease in the expression of genes involved in cell cycle regulation, such as cell division control protein 25 B (*Cdc25b*) and *Cdcd20* (Ge *et al.*, 2005a).

Evidence concerning the involvement of growth factors in the regulation of ILC proliferation is limited. IGF-1 is an example of a growth factor not only important for PLC proliferation but also playing a role in ILC proliferation. Deletion of the *Igf1* gene in mice decreases bromodeoxyuridine incorporation not only into PLC but also in ILC. Consequently testicular LC numbers are reduced in adulthood (Wang and Hardy, 2004). When *Igf1*^{-/-} mice with functional *Igf1* receptors are treated with recombinant IGF-1 the labelling indices of the PLC and ILC become similar to those of the age-matched wt mice, indicating that the reductions in labelling indices are regulated by IGF-1 (Hu et al., 2010) (Fig. 4).

LH has a clear stimulatory effect on ILC function and presumably does not have a direct effect on ILC proliferation, but acts indirectly through the stimulation of *Igf1* secretion (Closset et al., 1989). Support for this assumption comes from studies in *Igf1* knockout mice where LH is unable to stimulate LC proliferation, in contrast to wt mice (Wang and Hardy, 2004).

Mature ALC. By Day 50 of post-natal life the transformation of ILC into mature ALC has been completed in rodents. ALC are large cells mainly located in the centre of the interstitium. These cells with an oval to round nucleus are characterized by the abundant presence of SER, tubulovesicular mitochondria, and the absence of lipid droplets (reviewed in Mendis-Handagama and Ariyaratne, 2001). Unlike FLC, ALC are not surrounded by a basal membrane and an 'envelope' of fibroblasts. The cells are connected with adjacent ALC and fibroblasts by cell contacts (reviewed in Haider, 2004). With the disappearance of lipid droplets the mature ALC switches to a different source of cholesterol as steroid precursor; cholesterol is now obtained by *de novo* synthesis or from serum lipoproteins (Ge and Hardy, 2007). The dramatic reduction in the activity of the testosterone metabolizing enzymes *Srd5a1* and *Hsd3a*, and the concomitant high expression of *Hsd3b6* and *Hsd17b3*, the latter catalysing the conversion of androstenedione into testosterone, complete the mature androgen biosynthetic pathway (Fig. 2) (Ge and Hardy, 1998; O'Shaughnessy et al., 2000; Yamamura et al., 2014).

ALC express high levels and secrete large amounts of *Insl3* (Ivell et al., 2013). Several studies suggest that *Insl3* in the adult animal may exert a local effect in the testis, although not all data are conclusive. *In vitro* experiments have shown that *INSL-3* stimulates Leydig cell testosterone secretion via a cAMP-dependent mechanism (Pathirana et al., 2012; reviewed in Bay and Anand-Ivell, 2014). Another function of *Insl3* in the adult testis may be directed to germ cells. *In vitro* data suggest that *INSL-3* may help to protect meiotic germ cells from undergoing apoptosis (Kawamura et al., 2004). These observations are however corroborated by studies in a conditional knockout mouse model in which *Insl3* receptor gene *Rxfp2* ablation was specifically restricted to male germ cells; these mice fail to develop a testicular phenotype (Huang et al., 2012; reviewed in Bay and Anand-Ivell, 2014).

In contrast to PLC and ILC, glucocorticoids inhibit testosterone production in ALC (Ge et al., 1997). Initially it was thought that the switch from predominantly reductive to oxidative activity of the *Hsd11b1* enzyme was responsible for the metabolism of corticosterone in ALC thus protecting testosterone production against the negative effects of glucocorticoids (Ge et al., 1997). More recently the same group demonstrated the presence of type 2 11β -HSD (*Hsd11b2*) in ALC, although at a 1000-fold lower expression level than the type 1 enzyme. *Hsd11b2* is a high-affinity oxidase, while the type 1 isotope is a low-affinity oxidase.

The expression level of the latter enzyme may not be sufficient to protect ALC against stress levels of glucocorticoids. The presence of both enzyme isotypes in ALC is therefore thought to play an important physiological role in metabolizing glucocorticoids, thus further facilitating testosterone production (Ge et al., 2005b).

Although the expression of genes involved in cell cycle regulation and cell growth is very low in mature ALC (Ge et al., 2005a), interstitial cells in the adult testis continue to have the potency to proliferate. Support for this comes from a pulse-chase experiment performed several years before the cluster analysis by Ge et al. (2005a), showing that the interstitial cells in the adult testis still have a limited capacity to undergo cell renewal. In this study the turnover time of LC in the adult testis is determined to range from 142 days to the maximum life span of the animal, whereas the turnover time of the peritubular/myoid cells (including SLC) is estimated to range from 85 to 257 days (Teerds et al., 1989).

These are not the only studies suggesting that even in adulthood the LC lineage is flexible and dynamic. As indicated briefly, nearly 30 years ago it was discovered that the alkylating agent EDS selectively destroys the mature LC population in the adult rat testis (Kerr et al., 1985; Teerds et al., 1988). This process is followed by a complete regeneration of the original LC population within a few weeks after EDS administration. Later studies showed that EDS also acts as a LC toxicant in other species, such as mouse, guinea pig, hamster, monkey and rabbit. Evidence has become apparent that LC regeneration in the adult testis following EDS administration and the development of the ALC population in the (pre)pubertal testis take place along the same lineage (reviewed in Teerds and Rijntjes, 2007).

It is not the purpose of this review to present a complete overview of the similarities between SLC in the (pre)pubertal and adult testis. Only some highlights will be reported here. It is possible to isolate SLC from the adult testis following EDS administration, using the same methods previously used to isolate SLC from the post-natal day 7 testis (Ge et al., 2006; Stanley et al., 2012). These isolated cells express *Pdgfra* but not *Lhr* or the steroidogenic enzymes *Hsd3b* and *Cyp11a1*, like SLC in the post-natal testis. When 3 days after EDS administration seminiferous tubules with surrounding putative SLC are subjected to culture in the presence of LH and growth factors, these *Pdgfra* positive, *Hsd3b* negative cells develop during a subsequent 4-week culture period into *Hsd3b* positive cells that produce testosterone (Stanley et al., 2012).

The newly formed LC after EDS administration have many characteristics in common with PLC in the prepubertal testis, such as the frequent localization in close vicinity to seminiferous tubules, spindle-shaped morphology, and the expression of *Lhr* and *Hsd3b*. T3 administration stimulates the proliferation of these new LC (Ariyaratne et al., 2000b), while chronic hypothyroidism delays the formation of new LC after EDS administration (Rijntjes et al., 2010). These PLC in the adult testis subsequently differentiate into ILC, which like ILC in the pubertal testis have lipid droplets in their cytoplasm and produce up to 35 days after EDS significant levels of androstenediol. Between 35 and 76 days after EDS androstenediol levels gradually decrease (Vreeburg et al., 1988) while the testicular activity of both *Cyp11a1* and *Cyp17a1* increases, and as a consequence testosterone production further rises (O'Shaughnessy and Murphy, 1991). By Day 70 after EDS administration these ILC have differentiated into mature ALC, which no longer contain lipid droplets in their cytoplasm and have ceased to proliferate.

Recent evidence suggests that nerve growth factor (NGF) may be a potent regulator of ALC development, as it stimulates proliferation of

SLC, PLC and ILC following EDS administration (Zhang *et al.*, 2013). Whether NGF fulfils this role also in the (pre)pubertal testis remains to be elucidated.

Together, these data show that the interstitial cell population in the adult testis has under normal conditions a negligible proliferative capacity. However, when challenged the LC lineage appears to be highly dynamic with a great capacity for renewal.

LC development in the primate and rodent testis: similarities and differences

In this section we will give an overview of the most important similarities and differences between the LC lineage in primates and rodents.

In contrast to most mammalian species the development of human and primate LC is considered to be a tri-phasic process, consisting of fetal, neonatal and pubertal maturational phases (Nistal *et al.*, 1986; Prince, 2001, 2007). Both in rodents and primates the FLC population develops from stem cells, and as discussed above, presumably independent of gonadotrophic stimulation. In primates the FLC population however rapidly becomes highly dependent on placental hCG to stimulate testosterone production (Huhtaniemi *et al.*, 1979; Nistal *et al.*, 1986). The highest number of FLC and testosterone levels in the human testis is observed between gestational weeks 12 and 14 when masculinization occurs and placental hCG levels are still high (Holstein *et al.*, 1971; Codesal *et al.*, 1990). This is a major difference from the situation in rodents where even in the absence of pituitary LH masculinization proceeds normally during fetal life (Kendall *et al.*, 1995; Ma *et al.*, 2004).

When hCG levels decrease during the second trimester of human pregnancy, FLC numbers rapidly decline (Holstein *et al.*, 1971). The involution of the FLC population is associated with a reduction in cell volume, and signs of degeneration as depicted by the appearance of electron-dense cells and the presence of lipid accumulations (vacuoles) (Pelliniemi and Niemi, 1969; Holstein *et al.*, 1971; Codesal *et al.*, 1990). Some FLC escape involution and remain active throughout pregnancy (Codesal *et al.*, 1990; Prince, 2001). In the rodent testis regression of the FLC population largely occurs after birth (Tapanainen *et al.*, 1984).

Concomitant with the transient neonatal activation of the hypothalamic–pituitary–testicular axis between birth and 6 months postpartum, LC become prominent again in the human testis, which coincides with a sharp increase in testosterone production. The neonatal LC population consists of a mix of well-developed LC and smaller cells. These relatively large LC have ultrastructural features, such as extensive anastomosing SER tubules and pleomorphic mitochondria with tubular and lamellar cristae, well developed Golgi complex and regionalized rough endoplasmic reticulum, consistent with those of mature FLC, but also with ALC except that these neonatal LC do not have Reinke crystals in their cytoplasm (Prince, 1985, 1990, 2007). The smaller LC in the neonatal testis have a relatively underdeveloped morphology, e.g. prominent presence of cytoplasmic filaments, small mitochondria and moderate to relatively sparse SER, consistent with regressing LC. The large LC are considered to be responsible for the neonatal peak in testosterone production (Prince, 1990). This neonatal androgen surge may play a role in imprinting various cell types in androgen dependent organs, including the brain (reviewed in Svechnikov *et al.*, 2010).

There is consensus concerning the origin of the neonatal testosterone producing LC in the primate testis; these cells are thought to develop

through redifferentiation of involuted FLC as well as differentiation of stem/precursor cells (Codesal *et al.*, 1990; Prince, 2007; reviewed in Svechnikov *et al.*, 2010). LH plays an important role in the development and functioning of the neonatal LC population as blockade of the neonatal rise in LH levels in monkeys inhibits the formation of the neonatal testosterone producing LC population (Prince *et al.*, 1998). As discussed above, there is no evidence for the presence of a distinct neonatal LC population in rodents.

The neonatal androgen surge in primates is followed by a prolonged period of steroidogenic quiescence and inactivity of pituitary gonadotrophin secretion. The LC population consists now largely of regressed neonatal LC, also named infantile or immature LC, and LC precursors, which do not express steroidogenic enzymes like HSD3B (Verhagen *et al.*, 2014). These LC are characterized morphologically by convoluted nuclei, a small to moderate amount of SER and some lipid droplets in their cytoplasm; the stem cell/precursor cells have a more spindle-shaped morphology (Chemes, 1996; Prince, 2007). Although these cells are functionally inactive, acute hCG stimulation leads to a precocious rise in testosterone production, implicating that the cells can rapidly respond to a trophic stimulus (Chemes *et al.*, 1985). This so-called infantile period lasts until the onset of puberty.

Just before puberty when LH levels increase again HSD3B positive LC reappear in the primate testis, indicative of a steroidogenically active ALC population. The developing ALC undergo morphological changes, move from the peritubular region to the intertubular space and acquire an epithelioid shape. The SER becomes the most prominent cytoplasmic organelle forming a large network of anastomosing tubules hosting the enzymes involved in steroidogenesis. Mitochondria with tubular cristae and lamellar associations are observed in increasing numbers, as are well-developed Golgi elements, lipid droplets and Reinke crystals (Chemes, 1996; Prince, 2007). These morphological and functional changes are largely comparable to what is observed in the rodent testis with the formation of the ALC population. Both in rodents and primates the development and functioning of the ALC population is critically dependent on LH.

There are several hypotheses concerning the fate of the neonatal LC and the origin of the ALC population in the primate. One hypothesis postulates that the neonatal LC population largely undergoes involution; these cells do not express HSD3B and are thus functionally inactive (Nistal *et al.*, 1986). A limited number of neonatal LC may escape this fate and continue to be functionally active and responsible for the low testosterone levels in infancy (Swerdluff and Heber, 1981). The ALC population develops according to this hypothesis around puberty from stem/precursor cells mainly located in the vicinity of the seminiferous tubules (Nistal *et al.*, 1986; Rey *et al.*, 1996; Verhagen *et al.*, 2014). Another hypothesis suggests that the neonatal LC population undergoes partial involution and degeneration (Prince, 1984, 2001). The ALC population according to Prince develops from both involuted neonatal LC as well as differentiation of stem/precursor cells. A third hypothesis postulates that neonatal LC undergo partial involution during the infantile period of life. These cells disappear however before puberty, presumably around the time when ALC are formed by differentiation of stem/precursor cells (Nistal *et al.*, 1986). Proliferation of the newly formed ALC hardly contributes to the final size of the LC population in adulthood (Verhagen *et al.*, 2014), suggesting that in primates differentiation of stem/precursor cells and/or redifferentiation of involuted cells determine the size of the ALC population in adulthood. It remains to be elucidated which of these hypotheses is correct.

The ALC population in the primate testis has long been considered stable with negligible proliferative capacity. Nevertheless, there are indications that LC renewal does occur in the adult testis, not only after stimulation with supra-physiological doses of hCG (Christensen and Peacock, 1980; Teerds *et al.*, 1988), but also under normal conditions. Both in the human and monkey testis mitoses of mature ALC have been observed, although the frequency of LC proliferation is extremely low (Fouquet and Raynaud, 1985; Amat *et al.*, 1986; Fouquet and Khan, 1987). Considering the very low incidence of proliferation in the adult testis, this process is, like in the rodent, presumably not of physiological significance.

The studies in rodents have contributed enormously to our understanding of the essential steps in LC development and functioning, and the factors involved in these processes. Much less information is available about this process in humans and primates, although it is clear that there are differences of the former from rodents. Whether the same factors identified in rodents also play a role in human male LC development is at present largely unknown. Hopefully more advanced techniques will make it possible to increase our knowledge on these processes in the human/primate testis in the near future, which will increase the translational potential of this research.

LC function in aging: what can we learn from model animals

Testicular testosterone synthesis and secretion are under the control of the pituitary LH drive and paracrine modulators such as Igf1, cytokines and neurogenic signals. Aging in men is accompanied by a moderate gradual decrease in plasma testosterone levels and as a consequence, among others, by a decline in muscle mass and strength, bone density, sexual function, and increase in visceral adiposity (Perheentupa and Huhtaniemi, 2009; Huhtaniemi and Forti, 2011).

The age-related decrease in testosterone levels can be either due to a defect at the hypothalamic-pituitary level, or to a problem within the LC. Older reports attribute the age-related decrease in testosterone levels in males to a reduction in LC numbers (Neaves *et al.*, 1985; reviewed in Zaidi *et al.*, 2012). More recent endocrine literature suggests that this age-related decrease is largely due to a primary testicular failure (low testosterone and high LH levels) (reviewed in Perheentupa and Huhtaniemi, 2009). Veldhuis *et al.* (2012) reported in a study including 92 healthy men that, with aging, LH efficacy decreases, LC sensitivity to LH diminishes, and LH-testosterone down-regulation increases, implicating a reduced effectual LH drive as cause for decreased testosterone levels in older men. Whether the aging-related decline in LC function is an inevitable physiological phenomenon or a pathophysiological response to an aging-associated decline in general health is still a topic of debate. In support of the latter are the findings that old men in perfect physical condition show no decline in serum testosterone (Sartorius *et al.*, 2012), and that reversal of obesity, the main reason for low testosterone production in aging men, reverses testosterone suppression (Camacho *et al.*, 2013).

Ultrastructural analysis of LC in the aged human testis shows clear changes in morphology. The most frequently encountered alterations are poor development of mitochondria and SER, organelles involved in androgen biosynthesis, and accumulation of lipid droplets: the latter is considered a sign of functional impairment (Paniagua *et al.*, 1986). These morphological alterations support the observations of decreased LC functionality in the aged testis. Secondary hypofunction of the testis,

which is also regularly reported in older men (low testosterone and inappropriately normal or low LH levels), is largely independent of age and associated with obesity and comorbidities (Wu *et al.*, 2008, 2010).

In some strains of rodents there are indications that the age-related decrease in testosterone production is mainly due to a reduction in GnRH release from the hypothalamus, thus attenuating pituitary LH release (reviewed in Wang and Stocco, 2005). The age-related decrease in testosterone levels in these rodent models is therefore less well comparable to the situation in aging men, where the specific age effect attenuates LC function (Huhtaniemi and Forti, 2011). Reproductive aging in the Brown Norway rat has, in contrast to most other rodent species, solely a gonadal origin, which makes it a good model of human male aging. The testes of aged Brown Norway rats, when perfused with maximally stimulating doses of LH, produce significantly less testosterone than the testes of young rats (Zirkin *et al.*, 1993). This observation is confirmed by *in vitro* data showing that LC isolated from aged Brown Norway rat testes produce significantly less testosterone under LH stimulation than LC isolated from testes of young rats (Chen *et al.*, 2009). Due to the difficulty to obtain representative human male testicular tissue, it is not surprising that the Brown Norway rat is frequently used as a model for mechanistic studies of the age-related reduction in testicular testosterone production in humans (Chen *et al.*, 1994; reviewed in Zirkin and Tenover, 2012).

The production of steroid hormones is a complex process, which starts in LC either with *de novo* synthesis of cholesterol from acetate or acquisition of cholesterol from the cell membrane or extracellular sources through internalization of high or low density -cholesterol (depending on species). A rate-limiting step is the translocation of cholesterol from the cytoplasm to the vicinity of the Cyp11a1 enzyme located in the mitochondrial matrix. The transfer of cholesterol across the mitochondrial membranes is critically dependent on the presence of two proteins, StAR and translocator protein (Tspo) (reviewed in Midzak *et al.*, 2009); LH plays an important stimulatory role in this process. It activates the Gsa/cAMP/protein kinase A signalling cascade, leading to activation of transcription factors that regulate StAR gene expression as well as phosphorylation of the StAR protein. An age-related reduction in functioning has been observed in this regulatory cascade (reviewed in Wang and Stocco, 2005). Accumulation of recruited cholesterol in mitochondria is compromised in aged LC, arguing for an age-related decline in mitochondrial cholesterol transfer. This assumption is supported by the fact that both StAR and Tspo mRNA levels significantly diminish with age (Culty *et al.*, 2002).

Aging also negatively influences several enzymes that form the critical core of the steroidogenic pathway in LC. Reduced activity of Cyp11a1, Hsd3b, Cyp17a1, 17-ketoreductase, and Hsd11b2 has been reported in LC of aged Brown Norway and Lewis rats. Reductions in mRNA and protein expression of these steroidogenic enzymes (Luo *et al.*, 1996, 2005; Koeva *et al.*, 2009) fit well with the stereological analysis demonstrating a reduced absolute volume of mitochondria and a significant regression of microsomal volume in aged LC, limiting the maximal flux of steroid metabolites through the steroidogenic pathway (reviewed in Midzak *et al.*, 2009).

There is consensus that aging consists of a complex set of processes, involving numerous causes and consequences that all contribute to the deterioration of cellular functioning. Among these are changes in the metabolic generation of free radicals (Beattie *et al.*, 2013). According to the free radical theory, aging cells are in a chronic state of oxidative stress as a consequence of an age-related imbalance between

pro-oxidants and the antioxidant defence system. It is therefore not surprising that LC from testes of aged Brown Norway rats produce significantly larger amounts of reactive oxygen species (ROS) than cells from young rats (reviewed in Midzak *et al.*, 2009). Additionally, there may be deficits in the antioxidant defence system in LC. Microarray analysis has revealed an age-related down-regulation of several genes of the free radical scavenger family, including two glutathione transferase subunits, copper zinc superoxide dismutase (Sod1) – the most prominent antioxidant enzyme in LC, manganese superoxide dismutase (Sod2), and glutathione peroxidase (Gpx), leading to accumulation of free radicals (Luo *et al.*, 2006). A member of the ROS family, hydrogen peroxide, is capable of inhibiting steroidogenesis by interfering with cholesterol transport to the mitochondria and its conversion to pregnenolone by Cyp11a1 (Stocco *et al.*, 1993).

In line with these observations it has been shown that decreased steroidogenesis is associated with a deficiency in autophagy in aged rat LC (Li *et al.*, 2011). Autophagy is a cellular degenerative pathway that involves the delivery of affected cytoplasmic organelles to lysosomes, and is essential for cell survival, development and homeostasis (Mizushima and Levine, 2010). Li *et al.* (2011) show that decreased autophagic activity in aged LC may be responsible for the observed reduction in StAR expression and testosterone production, possibly by elevating the cellular ROS level.

Disruption at the level of cAMP signalling also appears to be involved in many of the age-related changes in LC testosterone production. A significant lesion in the ability of the Lhr to activate Gs protein and thereby adenyl cyclase and induce cAMP generation has been reported (reviewed in Midzak *et al.*, 2009). Two different possible explanations have been offered for this observation. In the first model the interaction of lipid peroxides generated from cellular ROS with membrane lipids results in lipid cross-linking, thus decreasing molecular mobility in the plasma membrane and, as a consequence, Lhr activation of Gs. In the second model the decreased Lhr coupling to Gs is explained by a redox shift to an oxidizing environment that can be linked to desensitization-type uncoupling of the receptor-adenyl cyclase interaction in aging LC (for further details, see Midzak *et al.*, 2009).

Based on the above observations one can conclude that, as aging itself, the age-related decrease in LC testosterone production is a multifactorial phenomenon. Although in some species decreased function of the hypothalamic-pituitary axis is reported, in humans and the model animal Brown Norway rat the major age-related defects occur within the LC. An age-related imbalance between ROS production and the antioxidant defence system, and the consequent oxidant-induced damage to LC is postulated to be a major player in the observed age-related reduction in steroidogenesis (Luo *et al.*, 2006).

Conclusions

Based on the above we can conclude that: the distinct populations of LC that develop along the life span of males include fetal, neonatal (in humans) and adult LC; although both in humans and rodents the formation of FLC is most likely independent of LH/CG stimulation, the newly formed FLC in the primate testis are highly dependent on CG for testosterone production, while in the mouse and rat FLC become LH-responsive later along the lineage. A plethora of paracrine factors within the fetal testis can apparently maintain (besides LH) FLC steroidogenesis in rodents, which in primates this is less obvious; albeit in rodents,

in contrast to primates, the initial steps of ALC development are not gonadotrophin sensitive due to the absence of Lhr, however the transition of SLC into PLC and the further development of the ALC population are highly dependent on LH; PLC, ILC and mature ALC in both the rodent and primate male differ from each other both morphologically and in their developmental and functional capacity; the age-related decrease in testosterone production in ALC is largely a consequence of defects in the steroidogenic pathway, presumably due to accumulation of ROS possibly in combination with a disturbed redox balance.

Authors' roles

I.T.H. is responsible for the Introduction and the section on 'Fetal Leydig cell development', the Conclusion, as well as Figs 1–3. K.J.T. is responsible for the Materials and Methods and the sections on 'Adult-type Leydig cell development', 'Comparative aspects between rodent and primate Leydig cell development' and the section on 'Aging'.

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Conflict of interest

The authors declare they are not aware of any conflict of interest.

References

- Adham IM, Steding G, Thamm T, Bullesbach EE, Schwabe C, Paprotta I, Engel W. The overexpression of the *insl3* in female mice causes descent of the ovaries. *Mol Endocrinol* 2002; **16**:244–252.
- Agoulnik AI. Relaxin and related peptides in male reproduction. *Adv Exp Med Biol* 2007; **612**:49–64.
- Albert O, Jegou B. A critical assessment of the endocrine susceptibility of the human testis to phthalates from fetal life to adulthood. *Hum Reprod Update* 2014; **20**: 231–249.
- Amat P, Paniagua R, Nistal M, Martin A. Mitosis in adult human Leydig cells. *Cell Tissue Res* 1986; **243**:219–221.
- Anand-Ivell R, Ivell R. Insulin-like factor 3 as a monitor of endocrine disruption. *Reproduction* 2014; **147**:R87–R95.
- Anand-Ivell R, Ivell R, Driscoll D, Manson J. Insulin-like factor 3 levels in amniotic fluid of human male fetuses. *Hum Reprod* 2008; **23**: 1180–1186.
- Anderson RA, Cambray N, Hartley PS, McNeilly AS. Expression and localization of inhibin alpha, inhibin/activin betaA and betaB and the activin type II and inhibin beta-glycan receptors in the developing human testis. *Reproduction* 2002; **123**:779–788.
- Ariyaratne HBS, Mendis-Handagama SMLC, Mason JI. Effects of tri-iodothyronine on testicular interstitial cells and androgen secretory capacity of the prepubertal rat. *Biol Reprod* 2000a; **63**:493–502.
- Ariyaratne HBS, Mills N, Mason JI, Mendis-Handagama SLMC. Effects of thyroid hormone on Leydig cell regeneration in the adult rat following ethane dimethane sulphonate treatment. *Biol Reprod* 2000b; **63**: 1115–1123.
- Baker PJ, Sha JA, McBride MW, Peng L, Payne AH, O'Shaughnessy PJ. Expression of 3 β -hydroxysteroid dehydrogenase type I and type VI isoforms in the mouse testis during development. *Eur J Biochem* 1999; **260**:911–916.
- Balvers M, Spiess AN, Domagalski R, Hunt N, Kilic E, Mukhupadhyay AK, Hanks E, Chartlon RM, Ivell R. Relaxin-like factor expression as a marker of differentiation in the mouse testis and ovary. *Endocrinology* 1998; **139**:2960–2970.
- Barsoum I, Yao HH. Redundant and differential roles of transcription factors Gli1 and Gli2 in the development of mouse fetal Leydig cells. *Biol Reprod* 2011; **84**:894–899.

- Barsoum IB, Kaur J, Ge RS, Cooke PS, Yao HH. Dynamic changes in fetal Leydig cell populations influence adult Leydig cell populations in mice. *FASEB J* 2013; **27**:2657–2666.
- Basciani S, Mariani S, Arizzi M, Ulisse S, Rucci N, Jannini EA, Della Rocca C, Manicone A, Carani C, Spera G et al. Expression of platelet-derived growth factor-A (PDGF-A), PDGF-B, and PDGF receptor- α and - β during human testicular development and disease. *J Clin Endocrinol Metab* 2002; **87**:2310–2319.
- Bay K, Anand-Ivell R. Human testicular insulin-like factor 3 and endocrine disrupters. *Vitam Horm* 2014; **94**:327–348.
- Bay K, Virtanen HE, Hartung S, Ivell R, Main KM, Skakkebaek NE, Andersson AM. Nordic Cryptorchidism Study Group, Toppari J. Insulin-like factor 3 levels in cord blood and serum from children: effects of age, postnatal hypothalamic-pituitary-gonadal axis activation, and cryptorchidism. *J Clin Endocrinol Metab* 2007; **92**:4020–4027.
- Beattie MC, Chen H, Fan J, Papadopoulos V, Miller P, Zirkin BR. Aging and luteinizing hormone effects on reactive oxygen species production and DNA damage in rat Leydig cells. *Biol Reprod* 2013; **88**:1–7.
- Behringer RR, Finegold MJ, Cate RL. Müllerian-inhibiting substance function during mammalian sexual development. *Cell* 1994; **79**:415–425.
- Bitgood MJ, McMahon AP. Hedgehog and Bmp genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo. *Dev Biol* 1995; **172**:126–138.
- Brennan J, Tilmann C, Capel B. Pdgfr- α mediates testis cord organization and fetal Leydig cell development in the XY gonad. *Genes Dev* 2003; **17**:800–810.
- Byskov AG. Differentiation of mammalian embryonic gonad. *Physiol Rev* 1986; **66**:71–117.
- Cailleau J, Vermeire S, Verhoeven G. Independent control of the production of insulin-like growth factor I and its binding protein by cultured testicular cells. *Mol Cell Endocrinol* 1990; **69**:79–89.
- Camacho EM, Huhtaniemi IT, O'Neill TW, Finn JD, Pye SR, Lee DM, Tajar A, Bartfai G, Boonen S, Casanueva FF et al.; EMAS Group. Age-associated changes in hypothalamic-pituitary-testicular function in middle-aged and older men are modified by weight change and lifestyle factors: longitudinal results from the European Male Ageing Study. *Eur J Endocrinol* 2013; **168**:445–455.
- Cattanach BM, Iddon CA, Charlton HM, Chiappa SA, Fink G. Gonadotrophin-releasing hormone deficiency in a mutant mouse with hypogonadism. *Nature* 1977; **269**:338–340.
- Chemes HE. Leydig cell development in humans. In: Payne AH, Hardy MP, Russell LD (eds). *The Leydig Cell*. Vienna, Illinois: Cache River Press, 1996, 175–202.
- Chemes HE, Gottlieb SE, Pasqualini T, Domenichini E, Rivarola MA, Bergada C. Response to acute hCG stimulation and steroidogenic potential of Leydig cell fibroblastic precursor cells in humans. *J Androl* 1985; **6**:102–112.
- Chen H, Hardy MP, Huhtaniemi I, Zirkin BR. Age-related decreased Leydig cell testosterone production in the Brown Norway rat. *J Androl* 1994; **15**:551–557.
- Chen H, Ge RS, Zirkin BR. Leydig cells: from stem cells to ageing. *Mol Cell Endocrinol* 2009; **306**:9–16.
- Chen H, Stanley E, Jin S, Zirkin BR. Stem Leydig cells: from fetal to aged animals. *Birth Defects Res C Embryo Today* 2010; **90**:272–283.
- Christensen AK, Peacock KC. Increase in Leydig cell number in testes of adult rats treated chronically with an excess of human chorionic gonadotropin. *Biol Reprod* 1980; **22**:383–391.
- Clark AM, Garland KK, Russell LD. Desert hedgehog (Dhh) gene is required in the mouse testis for formation of adult-type Leydig cells and seminiferous tubules. *Biol Reprod* 2000; **63**:1825–1838.
- Closset J, Gothot A, Sente B, Scippo ML, Igout I, Vandenbroeck M, Dombrowicz D, Hennen G. Pituitary hormones dependent expression of insulin-like growth factors I and II in the immature hypophysectomized rat testis. *Mol Endocrinol* 1989; **3**:1125–1131.
- Codesal J, Regadera J, Nistal M, Regadere-Sejas J, Paniagua R. Involution of human fetal Leydig cells. An immunohistochemical, ultrastructural and quantitative study. *J Anat* 1990; **172**:103–114.
- Cohen PF, Pollard JW. Normal sexual function in male mice lacking a functional type I interleukin-1 (IL-1) receptor. *Endocrinology* 1998; **39**:815–818.
- Cuducini C, Kercret H, Touzalin A-M, Ballet F, Jegou B. Vectorial production of interleukin 1 and interleukin 6 by rat Sertoli cells cultured in a dual compartment system. *Endocrinology* 1997; **38**:2863–2870.
- Culty M, Luo L, Yao ZX, Chen H, Papadopoulos V, Zirkin BR. Cholesterol transport, peripheral benzodiazepine receptor, and steroidogenesis in aging Leydig cells. *J Androl* 2002; **23**:439–447.
- DeFalco T, Takahashi S, Capel B. Two distinct origins for Leydig cell progenitors in the fetal testis. *Dev Biol* 2011; **352**:14–26.
- DeFalco T, Saraswathula A, Briot A, Iruela-Arispe ML, Capel B. Testosterone levels influence mouse fetal Leydig cell progenitors through Notch signalling. *Biol Reprod* 2013; **88**:91, 1–12.
- Dombrowicz D, Sente B, Closset J, Hennen G. Dose-dependent effects of human prolactin on the immature hypophysectomized rat testis. *Endocrinology* 1992; **130**:695–700.
- Dong L, Jelinsky SA, Finger JN, Johnston DS, Kopf GS, Sottas CM, Hardy MP, Ge R. Gene expression during development of fetal and adult Leydig cells. *Ann N Y Acad Sci* 2007; **1120**:16–35.
- El-Gehani F, Tena-Sempere M, Huhtaniemi I. Vasoactive intestinal peptide is an important endocrine regulatory factor of fetal rat testicular steroidogenesis. *Endocrinology* 1998; **139**:1474–1480.
- El-Gehani F, Tena-Sempere M, Huhtaniemi I. Evidence that pituitary adenylate cyclase-activating polypeptide is a potent regulator of fetal rat testicular steroidogenesis. *Biol Reprod* 2000; **63**:1482–1489.
- El-Gehani F, Tena-Sempere M, Ruskoaho H, Huhtaniemi I. Natriuretic peptides stimulate steroidogenesis in the fetal rat testis. *Biol Reprod* 2001; **65**:595–600.
- Flück CE, Miller WL, Auchus RJ. The 17, 20-lyase activity of cytochrome p450c17 from human fetal testis favors the delta5 steroidogenic pathway. *J Clin Endocrinol Metab* 2003; **88**:3762–3766.
- Flück CE, Meyer-Böni M, Pandey AV, Kempná P, Miller WL, Schoenle EJ, Bignon-Laubert A. Why boys will be boys: two pathways of fetal testicular androgen biosynthesis are needed for male sexual differentiation. *Am J Hum Genet* 2011; **89**:201–218.
- Fouquet J-P, Khan M-L. Mitotic activity in monkey and rat Leydig cells. *Reprod Nutr Dev* 1987; **27**:1053–1062.
- Fouquet J-P, Raynaud F. Renewal of Leydig cells in the neonatal and adult monkey: a radioautographic study. *Biol Cell* 1985; **54**:187–190.
- Fowler PA, Cassie S, Rhind SM, Brewer MJ, Collinson JM, Lea RG, Baker PJ, Bhattacharya S, O'Shaughnessy PJ. Maternal smoking during pregnancy specifically reduces human fetal desert hedgehog gene expression during testis development. *J Clin Endocrinol Metab* 2008; **93**:619–626.
- Fowler PA, Bhattacharya S, Gromoll J, Monteiro A, O'Shaughnessy PJ. Maternal smoking and developmental changes in luteinizing hormone (LH) and the LH receptor in the fetal testis. *J Clin Endocrinol Metab* 2009; **94**:4688–4695.
- Franco HL, Yao HH. Sex and hedgehog: roles of genes in the hedgehog signaling pathway in mammalian sexual differentiation. *Chromosome Res* 2012; **20**:247–258.
- Gangnerau MN, Picon R. Onset of steroidogenesis and differentiation of functional LH receptors in rat fetal testicular cultures. *Arch Androl* 1987; **18**:215–224.
- Gaytan F, Bellido C, Aguilar E, van Rooijen N. Requirement for testicular macrophages in Leydig cell proliferation and differentiation during prepubertal development in rats. *J Reprod Fertil* 1994; **102**:93–399.
- Ge RS, Hardy MP. Decreased cyclin A2 and increased cyclin G1 levels coincide with loss of proliferative capacity in rat Leydig cells during pubertal development. *Endocrinology* 1997; **138**:3719–3726.
- Ge R-S, Hardy MP. 1998 Variation in the end products of androgen biosynthesis and metabolism during postnatal differentiation of rat Leydig cells. *Endocrinology* 1998; **139**:3787–3795.
- Ge R, Hardy MP. Regulation of Leydig cells during pubertal development. In: Payne AH, Hardy MP (eds). *The Leydig Cell in Health and Disease*. Totowa, New Jersey: Humana Press, 2007, 55–70.
- Ge R, Hardy DO, Catterall JF, Hardy MP. Developmental changes in glucocorticoid receptor and 11 β -hydroxysteroid dehydrogenase oxidative and reductive activities in rat Leydig cells. *Endocrinology* 1997; **138**:5089–5095.
- Ge RS, Hardy DO, Catterall JF, Hardy MP. Opposing changes in 3 β -hydroxysteroid dehydrogenase oxidative and reductive activities in rat Leydig cells during pubertal development. *Biol Reprod* 1999; **60**:855–860.
- Ge R, Dong Q, Sottas CM, Chen H, Zirkin BR, Hardy MP. Gene expression in rat Leydig cells during development from the progenitor to adult stage: a cluster analysis. *Biol Reprod* 2005a; **72**:1405–1415.
- Ge RS, Dong Q, Niu E-M, Sottas CM, Hardy DO, Catterall JF, Latif SA, Morris DJ, Hardy MP. 11 β -Hydroxysteroid dehydrogenase 2 in rat Leydig cells: its role in blunting glucocorticoid action at physiological levels of substrate. *Endocrinology* 2005b; **146**:2657–2664.

- Ge R, Dong Q, Sottas CM, Papadopoulos V, Zirkin BR, Hardy MP. In search of the rat stem Leydig cells: identification, isolation, and lineage specific development. *Proc Natl Acad Sci USA* 2006; **103**:2719–2724.
- Gnessi L, Basciani S, Mariani S, Arizzi M, Spera G, Wang C, Bondjers C, Karlsson L, Betsholtz C. Leydig cell loss and spermatogenesis arrest in platelet-derived growth factor (PDGF)-A deficient mice. *J Cell Biol* 2000; **149**:1019–1026.
- Gondos B. Development and differentiation of the testis and male reproductive tract. In: Steinberger ASE (ed). *Testicular Development, Structure and Function*. New York: Raven Press, 1980, 3–20.
- Gorlov IP, Kamat A, Bogatcheva NV, Jones E, Lamb DJ, Truong A, Bishop CE, McElreavey K, Agoulnik AI. Mutations of the GREAT gene cause cryptorchidism. *Hum Mol Genet* 2002; **11**:2309–2318.
- Greco TL, Payne AH. Ontogeny of expression of the genes for steroidogenic enzymes P450 side-chain cleavage, 3 beta-hydroxysteroid dehydrogenase, P450 17 alpha-hydroxylase/C17–20 lyase, and P450 aromatase in fetal mouse gonads. *Endocrinology* 1994; **135**:262–268.
- Griswold SL, Behringer RR. Fetal Leydig cell origin and development. *Sex Dev* 2009; **3**:1–15.
- Guo JJ, Ma X, Wang CCQF, Ge Y-F, Lian Q-Q, Hardy DO, Zhang YF, Dong Q, Xu YF, Ge R-S. Effects of luteinizing hormone and androgen on the development of rat progenitor Leydig cells *in vitro* and *in vivo*. *Asian J Androl* 2012; **15**:685–691.
- Hacker A, Capel B, Goodfellow P, Lovell-Badge R. Expression of Sry, the mouse sex determining gene. *Development* 1995; **121**:1603–1614.
- Haider SG. Cell biology of Leydig cells in the testis. *Int Rev Cytol* 2004; **233**:181–241.
- Hallmark N, Walker M, McKinnel C, Mahood IK, Scott H, Bayne R, Coutts S, Anderson RA, Greig I, Morris K *et al*. Effects of monobutyl and di(n-butyl) phthalate *in vitro* on steroidogenesis and Leydig cell aggregation in fetal testis explants from the rat: comparison with effects *in vivo* in the fetal rat and neonatal marmoset and *in vitro* in the human. *Environ Health Perspect* 2007; **115**:390–396.
- Handelsman DJ, Spaliviero JA, Scott CD, Baxter RC. Identification of insulin-like growth factor-I and its receptors in the rat testis. *Acta Endocrinol (Copenh)* 1985; **109**:543–549.
- Hardy MP, Zirkin BR, Ewing LL. Kinetic studies on the development of the adult population of Leydig cells in the testis of pubertal rat. *Endocrinology* 1989; **124**:762–770.
- Hatano O, Takakusu A, Nomura M, Morohashi K. Identical origin of adrenal cortex and gonad revealed by expression profiles of Ad4BP/SF-1. *Genes Cells* 1996; **1**:663–671.
- Hazra R, Jimenez M, Desai R, Handelsman DJ, Allan CM. Sertoli cell androgen receptor expression regulates temporal fetal and adult Leydig cell differentiation, function, and population size. *Endocrinology* 2013; **154**:3410–3422.
- Heger NE, Hall SJ, Sandrof MA, McDonnell EV, Hensley JB, McDowell EM, Martin KA, Gaido KW, Johnson KJ, Boekelheide K. Human fetal testis xenografts are resistant to phthalate-induced endocrine disruption. *Environ Health Perspect* 2012; **120**:1137–1143.
- Holstein AF, Wartenberg H, Vosmeyer J. Zur Cytologie der pränatalen Gonadenentwicklung beim Menschen. III Die Entwicklung der Leydigzellen im Hoden von Embryonen und Feten. *Z Anat Entwickl-Gesch* 1971; **135**:43–66.
- Hu G-X, Lin H, Chen G-R, Chen B-B, Lian Q-Q, Hardy DO, Zirkin BR, Ge R-S. Deletion of the *Igf1* gene: suppressive effects of adult Leydig cell development. *J Androl* 2010; **31**:379–387.
- Huang Z, Rivas B, Agoulnik AI. Insulin-like 3 signaling is important for testicular descent but dispensable for spermatogenesis and germ cell survival in adult mice. *Biol Reprod* 2012; **87**:143.
- Huhtaniemi I, Forti G. Male late-onset hypogonadism: pathogenesis, diagnosis and treatment. *Nat Rev Urol* 2011; **8**:335–344.
- Huhtaniemi I, Ilkonen M, Vihko R. Presence of testosterone and other neutral steroids in human fetal testes. *Biochem Biophys Res Commun* 1970; **38**:715–720.
- Huhtaniemi IT, Korenbrot CC, Jaffe RB. HCG binding and stimulation of testosterone biosynthesis in the human fetal testis. *J Clin Endocrinol Metab* 1977; **44**:963–967.
- Huhtaniemi I, Korenbrot C, Lautala P, Jaffe RB. Testicular steroidogenesis and its regulation in the primate fetus and newborn. *Ann Biol Anim Biochim Biophys* 1979; **19**:1327–1338.
- Ivell R, Bathgate RAD. Insulin-like peptide 3 in Leydig cells. In: Payne AH, Hardy MP (eds). *The Leydig Cell in Health and Disease*. Totowa, New Jersey: Humana Press, Inc., 2007, 279–289.
- Ivell R, Balvers M, Anand RJK, Paust H-J, McKinnel C, Sharpe R. Differentiation-dependent expression of 17 β -hydroxysteroid dehydrogenase type 10, in the rodent testis: effect of aging in Leydig cells. *Endocrinology* 2003; **144**:3130–3137.
- Ivell R, Wade JD, Anand-Ivell R. INSL3 as a biomarker of Leydig cell functionality. *Biol Reprod* 2013; **88**:147. 1–148.
- Japon MA, Rubinstein M, Low MJ. *In situ* hybridization analysis of anterior pituitary hormone gene expression during fetal mouse development. *J Histochem Cytochem* 1994; **42**:1117–1125.
- Johnson KJ, Heger NE, Boekelheide K. Of mice and men (and rats): phthalate-induced fetal testis endocrine disruption is species-dependent. *Toxicol Sci* 2012; **129**:235–248.
- Kanzaki M, Morris PL. Growth hormone regulates steroidogenic acute regulatory protein expression and steroidogenesis in Leydig cell progenitors. *Endocrinology* 1999; **140**:1681–1686.
- Kawamura K, Kumagi J, Sudo S, Chun SY, Pisarska M, Morita H, Toppari J, Fu P, Wade JD, Bathgate RA *et al*. Paracrine regulation of mammalian oocyte maturation and male germ cell survival. *Proc Natl Acad Sci USA* 2004; **101**:7323–7328.
- Keeney DS, Jenkins CM, Waterman MR. Developmentally regulated expression of adrenal 17 alpha-hydroxylase cytochrome P450 in the mouse embryo. *Endocrinology* 1995; **136**:4872–4879.
- Kendall SK, Samuelson LC, Saunders TL, Wood RI, Camper SA. Targeted disruption of the pituitary glycoprotein hormone alpha-subunit produces hypogonadal and hypothyroid mice. *Genes Dev* 1995; **9**:2007–2019.
- Kerr JB. Functional cytology of the human testis. *Ballière's Clin Endocrinol Metab* 1992; **6**:235–250.
- Kerr J, Donachie FK, Rommerts FFG. Selective destruction and regeneration of rat Leydig cells *in vivo*: a new method for the study of seminiferous tubular-interstitial tissue interaction. *Cell Tissue Res* 1985; **242**:145–156.
- Ketola I, Pentikäinen V, Vaskivuo T, Ilvesmäki V, Herva R, Dunkel L, Tapanainen JS, Toppari J, Heikinheimo M. Expression of transcription factor GATA-4 during human testicular development and disease. *J Clin Endocrinol Metab* 2000; **85**:3925–3931.
- Khan SA, Khan SJ, Dorrington JH. Interleukin-1 stimulates deoxyribonucleic acid synthesis in immature rat Leydig cells *in vitro*. *Endocrinology* 1992a; **131**:1853–1857.
- Khan S, Teerds K, Dorrington J. Growth factor requirements for DNA synthesis by Leydig cells from the immature rat. *Biol Reprod* 1992b; **46**:335–341.
- Khan SA, Mirsafian M, Howdeshell K, Dorrington JH. Transforming growth factor- β inhibits DNA synthesis in immature rat Leydig cells *in vitro*. *Mol Cell Endocrinol* 1999; **48**:21–28.
- Kilcoyne KR, Smith LB, Atanassova N, McPherson S, McKinnel C, van den Driesche S, Jobling MS, Chambers TJG, De Gendt K, Verhoeven G *et al*. Fetal programming of adult Leydig cell function by androgenic effects of stem/progenitor cells. *Proc Natl Acad Sci USA* 2014; **111**:E1924–E1932.
- Kitamura K, Yanazawa M, Sugiyama M, Miura H, Iizuka-Kogo A, Kusaka M, Omichi K, Kato-Fukui Y, Kamiiriza K, Matsuo M *et al*. Mutation of ARX causes abnormal development of forebrain and testes in mice and X-linked lissencephaly with abnormal genitalia in humans. *Nat Genet* 2002; **32**:359–369.
- Koeva Y, Bakalska M, Atanassova N, Georgieva K, Davidoff M. Age-related changes in the expression of 17 β -hydroxysteroid dehydrogenase type 2 in rat Leydig cells. *Folia Histochem Cytobiol* 2009; **47**:281–287.
- Koskimies P, Suvanto M, Nokkala E, Huhtaniemi IT, McLuskey A, Themmen APN, Poutanen M. Female mice carrying a ubiquitin promoter-Insl3 transgene have descended ovaries and inguinal hernias but normal fertility. *Mol Cell Endocrinol* 2003; **206**:159–166.
- Kremer H, Kraaij R, Toledo SPA, Post M, Fridman JB, Hayashida CY, van Reen M, Milgrom E, Ropers H-H, Mariman E *et al*. Male pseudohermaphroditism due to a homozygous missense mutation of the luteinizing hormone receptor gene. *Nat Genet* 1995; **9**:160–164.
- Krishnan V, Pereira FA, Qiu Y, Chen CH, Beachy PA, Tsai SY, Tsai MJ. Mediation of Sonic hedgehog-induced expression of COUP-TFII by a protein phosphatase. *Science* 1997; **278**:1947–1950.
- Kroft TL, Patterson J, Won Yoon J, Doglio L, Walterhouse DO, Iannaccone PM, Goldberg E. GLII localization in the germinal epithelial cells alternates between cytoplasm and nucleus: upregulation in transgenic mice blocks spermatogenesis in pachytene. *Biol Reprod* 2001; **65**:1663–1671.
- Kumagai J, Hsu SY, Matsumi H, Roh JS, Fu P, Wade JD, Bathgate RA, Hsueh AJ. INSL3/Leydig insulin-like peptide activates the LGR8 receptor important in testis descent. *J Biol Chem* 2002; **277**:31283–31286.
- Lei ZM, Mishra S, Ponnuru P, Li X, Wang ZW, Rao ChV. Testicular phenotype in luteinizing hormone receptor knockout animals and the effect of testosterone replacement. *Biol Reprod* 2004; **71**:605–1613.

- Li W-R, Chen L, Chang ZJ, Xin H, Liu T, Zhang Y-Q, Li YG, Zhou F, Gong Y-Q, Gao ZZ *et al.* Autophagy deficiency is related to steroidogenic decline in aged rat Leydig cells. *Asian J Androl* 2011; **13**:881–888.
- Lofrano-Porto AGB, Barra GB, Giacomini LA, Nascimento PP, Latronico AC, Casulari LA, Neves FDADR. Luteinizing hormone beta mutation and hypogonadism in men and women. *N Engl J Med* 2007; **357**:897–904.
- Luo L, Chen H, Zirkin BR. Are Leydig cell steroidogenic enzymes differentially regulated with aging? *J Androl* 1996; **17**:509–515.
- Luo L, Chen H, Zirkin BR. Temporal relationships among testosterone production, steroidogenic acute regulatory protein (StAR), and P450 side-chain cleavage enzyme (P450_{scc}) during Leydig cell aging. *J Androl* 2005; **26**:25–31.
- Luo L, Chen H, Trush MA, Show MD, Anway MD, Zirkin BR. Aging and the brown Norway rat Leydig cells antioxidant defense system. *J Androl* 2006; **27**:240–247.
- Lyet L, Louis F, Forest MG, Jossen N, Behringer RR, Vigier B. Ontogeny of reproductive abnormalities induced by deregulation of anti-Müllerian hormone expression in transgenic mice. *Biol Reprod* 1995; **52**:444–454.
- Ma X, Dong Y, Matzuk MM, Kumar TR. Targeted disruption of luteinizing hormone beta-subunit leads to hypogonadism, defects in gonadal steroidogenesis, and infertility. *Proc Natl Acad Sci USA* 2004; **101**:17294–17299.
- Mahendroo M, Wilson JD, Richardson JA, Auchus RJ. Steroid 5 α -reductase 1 promotes 5 α -androstane-3 α ,17 β -diol synthesis in immature mouse testes by two pathways. *Mol Cell Endocrinol* 2004; **222**:113–120.
- Majdic G, Saunders PT, Teerds KJ. Immunoeexpression of the steroidogenic enzymes 3- β -hydroxysteroid dehydrogenase and 17 α -hydroxylase, C17,20 lyase and the receptor for luteinizing hormone (LH) in the fetal rat testis suggests that the onset of Leydig cell steroid production is independent of LH action. *Biol Reprod* 1998; **58**:520–525.
- Marians RC, Ng L, Blair HC, Unger P, Graves PN, Davies TF. Defining thyrotropin-dependent and -independent steps of thyroid hormone synthesis by using thyrotropin receptor-null mice. *Proc Natl Acad Sci USA* 2002; **99**:15776–15781.
- Martinez-Arguelles DB, Campioli E, Culty M, Zirkin BR, Papadopoulos V. Fetal origin of endocrine dysfunction in the adult: the phthalate model. *J Steroid Biochem Mol Biol* 2013; **137**:5–17.
- Mathur RS, Wigvist N, Diczfalusy E. De novo synthesis of steroids and steroid sulphates by the testicles of the human foetus at midgestation. *Acta Endocrinol (Copenh)* 1972; **71**:792–800.
- Mazaud-Guittot S, Nicolaz CN, Desdoits-Lethimonier C, Coiffec I, Maamar MB, Balaguer P, Kristensen DM, Chevrier C, Lavoué V, Poulain P *et al.* Paracetamol, aspirin, and indomethacin induce endocrine disturbances in the human fetal testis capable of interfering with testicular descent. *J Clin Endocrinol Metab* 2013; **98**:E1757–E1767.
- McDowell EN, Kisielewski AE, Pike JW, Franco HL, Yao HH-C, Johnson KJ. A transcriptome-wide screen for mRNAs enriched in fetal Leydig cells: CRHR1 agonism stimulates rat and mouse fetal testis steroidogenesis. *PLoS One* 2012; **7**:e47359. doi:10.1371/journal.pone.0047359.
- McKinnell C, Sharpe RM, Mahood K, Hallmark N, Scott H, Ivell R, Staub C, Jégou B, Haag F, Koch-Nolte F *et al.* Expression of insulin-like factor 3 protein in the rat testis during fetal and postnatal development and in relation to cryptorchidism induced by in utero exposure to di (n-Butyl) phthalate. *Endocrinology* 2005; **146**:4536–4544.
- Mendis-Handagama SMLC, Ariyaratne HBS. Differentiation of the adult Leydig cell population in the postnatal testis. *Biol Reprod* 2001; **65**:660–671.
- Mendis-Handagama SMLC, Ariyaratne HBS, Teunissen van Manen KR, Haupt RL. Differentiation of adult Leydig cells in the neonatal rat testis is arrested by hypothyroidism. *Biol Reprod* 1998; **59**:351–357.
- Midzak AS, Chen H, Papadopoulos V, Zirkin BR. Leydig cell aging and the mechanism of reduced testosterone synthesis. *Mol Cell Endocrinol* 2009; **299**:23–31.
- Mitchell RT, Childs AJ, Anderson RA, van den Driesche S, Saunders PT, McKinnell C, Wallace WH, Kelnar CJ, Sharpe RM. Do phthalates affect steroidogenesis by the human fetal testis? Exposure of human fetal testis xenografts to di-n-butyl phthalate. *J Clin Endocrinol Metab* 2012; **97**:e341–e348.
- Mitchell RT, Sharpe RM, Anderson RA, McKinnell C, Macpherson S, Smith LB, Wallace WHB, Kelnar CJH, van den Driesche S. Diethylstilboestrol exposure does not reduce testosterone production in human fetal testis xenografts. *PLoS One* 2013; **8**:e61726.
- Miyabayashi K, Katoh-Fukui Y, Ogawa H, Baba T, Shima Y, Sugiyama N, Kitamura K, Morohashi K-I. Aristaless related homeobox gene, *Arx*, is implicated in mouse fetal Leydig cell differentiation possibly through expressing in the progenitor cells. *PLoS One* 2013; **8**:e68050.
- Miyado M, Nakamura M, Miyado K, Morohashi K, Sano S, Nagata E, Fukami M, Ogata T. Maml1 deficiency significantly reduces mRNA expression levels of multiple genes expressed in mouse fetal Leydig cells but permits normal genital and reproductive development. *Endocrinology* 2012; **153**:6033–6040.
- Mizushima N, Levine B. Autophagy in mammalian development and differentiation. *Nat Cell Biol* 2010; **12**:823–830.
- Moody S, Goh H, Bielanowicz A, Rippon P, Loveland KL, Itman C. Prepubertal mouse testis growth and maturation and androgen production are acutely sensitive to di-n-butyl phthalate. *Endocrinology* 2013; **154**:3460–3475.
- Mullaney BP, Skinner MK. Transforming growth factor- α and epidermal growth factor gene expression and action during pubertal development of the seminiferous tubule. *Mol Endocrinol* 1992; **6**:2103–2113.
- Neaves WB, Johnson L, Petty CS. Age-related change in numbers of other interstitial cells in testes of adult men: evidence bearing on the fate of Leydig cells lost with increasing age. *Biol Reprod* 1985; **33**:259–269.
- Nef S, Parada LF. Cryptorchidism in mice mutant for *Ins13*. *Nat Genet* 1999; **22**:295–299.
- Nistal M, Paniagua R, Regadera J, Santamaria L, Amat P. A quantitative morphological study of human Leydig cells from birth to adulthood. *Cell Tissue Res* 1986; **246**:229–236.
- N'Tumba-Byn T, Moison D, Lacroix M, Lecureuil C, Lesage L, Prud'homme SM, Pozzi-Gaudin S, Frydman R, Benachi A, Livera G *et al.* Differential effects of bisphenol A and diethylstilbestrol on human, rat and mouse fetal Leydig cell function. *PLoS One* 2012; **7**:e51579.
- Nusslein-Volhard C, Wieschaus E. 1980 Mutations affecting segment number and polarity in *Drosophila*. *Nature* 1980; **287**:795–801.
- Ogata T, Sano S, Nagata E, Kato F, Fukami M. MAMLD1 and 46, XY disorders of sex development. *Semin Reprod Med* 2012; **30**:410–416.
- Ormandy CJ, Camus A, Barra J, Damotte D, Lucas B, Buteau H, Edery M, Brousse N, Babinet C, Binart N *et al.* Null mutation of the prolactin receptor gene produces multiple reproductive defects in the mouse. *Genes Dev* 1997; **11**:167–178.
- O'Shaughnessy PJ, Murphy L. Steroidogenic enzyme activity in the rat testis following Leydig cell destruction by ethylene-1,2-dimethanesulphonate and subsequent Leydig cell regeneration. *J Endocrinol* 1991; **131**:451–457.
- O'Shaughnessy PJ, Baker P, Sohnius U, Haavisto AM, Charlton HM, Huhtaniemi I. Fetal development of Leydig cell activity in the mouse is independent of pituitary gonadotroph function. *Endocrinology* 1998; **139**:1141–1146.
- O'Shaughnessy PJ, Baker PJ, Heikkilä M, Vainio S, McMahon AP. Localization of 17 β -hydroxysteroid dehydrogenase/17-ketosteroid reductase isoform expression in the developing mouse testis-androstenedione is the major androgen secreted by fetal/neonatal leydig cells. *Endocrinology* 2000; **141**:2631–2637.
- O'Shaughnessy PJ, Flemming LM, Jackson G, Hochgeschwender U, Reed P, Baker PJ. Adrenocorticotrophic hormone directly stimulates testosterone production by the fetal and neonatal mouse testis. *Endocrinology* 2003; **144**:3279–3284.
- O'Shaughnessy PJ, Baker PJ, Monteiro A, Cassie S, Bhattacharya S, Fowler PA. Developmental changes in human fetal testicular cell numbers and messenger ribonucleic acid levels during the second trimester. *J Clin Endocrinol Metab* 2007; **92**:4792–4801.
- O'Shaughnessy PJ, Morris ID, Huhtaniemi I, Baker PJ, Abel MH. Role of androgen and gonadotrophins in the development and function of the Sertoli cells and Leydig cells: data from mutant and genetically modified mice. *Mol Cell Endocrinol* 2009; **306**:2–8.
- Overbeek PA, Gorlov IP, Sutherland RW, Houston JB, Harrison VWR, Boettger-Tong HL, Bishop CE, Agoulnik AI. A transgenic insertion causing cryptorchidism in mice. *Genesis* 2011; **30**:26–35.
- Pakarinen P, Kimura S, El-Gehani F, Pelliniemi LJ, Huhtaniemi I. Pituitary hormones are not required for sexual differentiation of male mice: phenotype of the *T/ebp/Nkx2.1* null mutant mice. *Endocrinology* 2002; **143**:4477–4482.
- Paniagua R, Amat P, Nistal M, Martin A. Ultrastructure of Leydig cells in the human ageing testes. *J Anat* 1986; **146**:173–183.
- Park SY, Meeks JJ, Raverot G, Pfaff LE, Weiss J, Hammer GD, Jameson JL. Nuclear receptors Sfl and Dax1 function cooperatively to mediate somatic cell differentiation during testis development. *Development* 2005; **132**:2415–2423.
- Park SY, Tong M, Jameson JL. Distinct roles for steroidogenic factor 1 and desert hedgehog pathways in fetal and adult Leydig cell development. *Endocrinology* 2007; **148**:3704–3710.

- Pathirana IN, Kawate N, Büllsbach EE, Takahashi M, Hatoya S, Inaba T, Tamada H. Insulin-like peptide 3 stimulates testosterone secretion in mouse Leydig cells via cAMP pathway. *Regul Pept* 2012; **178**:102–106.
- Pelliniemi LJ, Niemi M. Fine structure of the human foetal. I. The interstitial tissue. *Cell Tissue Res* 1969; **99**:507–522.
- Perheentupa A, Huhtaniemi I. Aging of the human ovary and testis. *Mol Cell Endocrinol* 2009; **299**:2–13.
- Poling MC, Kauffman AS. Sexually dimorphic testosterone secretion in prenatal and neonatal mice is independent of kisspeptin-Kiss1r and GnRH signaling. *Endocrinology* 2012; **153**:782–793.
- Prince FP. Ultrastructure of immature Leydig cells in the human prepubertal testis. *Anat Rec* 1984; **209**:165–176.
- Prince FP. An ultrastructural and immunoperoxidase study of the neonatal human testis: evidence of mature, testosterone producing Leydig cells and Leydig cell regression. *Anat Rec* 1985; **211**:156A.
- Prince FP. Ultrastructural evidence of mature Leydig cells and Leydig cell regression in the neonatal human testis. *Anat Rec* 1990; **228**:405–417.
- Prince FP. The triphasic nature of Leydig cell development in humans, and comments on nomenclature. *J Endocrinol* 2001; **168**:213–216.
- Prince FP. The Human Leydig Cell. In: Payne AH, Hardy MP (eds). *The Leydig Cell in Health and Disease*. Totowa, New Jersey: Humana Press, 2007, 71–89.
- Prince FP, Mann DR, Fraser HM. Blockade of the hypothalamic-pituitary-testicular axis with a GnRH antagonist in the neonatal marmoset monkey: changes in Leydig cell ultrastructure. *Tissue Cell* 1998; **30**:651–661.
- Qin J, Tsai MJ, Tsai SY. Essential roles of COUP-TFII in Leydig cell differentiation and male fertility. *PLoS One* 2008; **3**:e3285.
- Racine C, Rey R, Forest MG, Louis F, Ferré A, Huhtaniemi I, Josso N, di Clemente N. Receptors for anti-Müllerian hormone on Leydig cells are responsible for its effects on steroidogenesis and cell differentiation. *Proc Natl Acad Sci USA* 1998; **95**:594–599.
- Rakoczy J, Fernandez-Valverde SL, Glazov EA, Wainwright EN, Sato T, Takada S, Combes AN, Korbic DJ, Miller D, Grimmond SM et al. MicroRNAs-140-5p/140-3p modulate Leydig cell numbers in the developing mouse testis. *Biol Reprod* 2013; **88**:143.
- Rey RA, Nagle CA, Chemes H. Morphometric study of the testicular interstitial tissue of the monkey *Cebus apella* during postnatal development. *Tissue Cell* 1996; **28**:31–42.
- Ricci G, Guglichmo MC, Caruso M, Ferranti F, Canipari R, Galdieri M, Catzone A. Hepatocyte growth factor is a mouse fetal Leydig cell terminal differentiation factor. *Biol Reprod* 2012; **87**:146, 1–11.
- Rijntjes E, Swarts HJM, Anand-Ivell R, Teerds KJ. Prepubertal induced chronic dietary hypothyroidism delays but does not block adult-type Leydig cell development. *Am J Physiol Endocrinol Metab* 2009; **296**:E305–E314.
- Rijntjes E, van Kesteren-Buiting A, Keijer J, Teerds KJ. Chronic hypothyroidism only marginally affects adult-type Leydig cell regeneration after EDS administration. *Int J Androl* 2010; **33**:e123–e131.
- Rouiller-Fabre V, Lecref L, Gautier C, Saez JM, Habert R. Expression and effect of insulin-like growth factor I on rat fetal Leydig cell function and differentiation. *Endocrinology* 1998; **139**:2926–2934.
- Sadeghian H, Anand-Ivell R, Balvers M, Relan V, Ivell R. Constitutive regulation of the *Insl3* gene in rat Leydig cells. *Mol Cell Endocrinol* 2005; **241**:10–20.
- Salva A, Hardy MP, Wu X-F, Sottas CM, MacLaughlin DT, Donahoe PK, Lee MM. Müllerian-inhibiting substance inhibits rat Leydig cell regeneration after ethylene dimethanesulphonate ablation. *Biol Reprod* 2004; **70**:600–607.
- Sarraj MA, Escalona RM, Umbers A, Chua HK, Small C, Griswold M, Loveland K, Findlay JK, Stenver KJ. Fetal testis dysgenesis and compromised Leydig cell function in Tgfb3 (beta glycan) knockout mice. *Biol Reprod* 2010; **82**:153–162.
- Sartorius G, Spasevska S, Idan A, Turner L, Forbes E, Zamojska A, Allan CA, Ly LP, Conway AJ, McLachlan RI et al. Serum testosterone, dihydrotestosterone and estradiol concentrations in older men self-reporting very good health: the healthy man study. *Clin Endocrinol (Oxf)* 2012; **77**:755–763.
- Scott HM, Mason JI, Sharpe RM. Steroidogenesis in the fetal testis and its susceptibility to disruption by exogenous compounds. *Endocr Rev* 2009; **30**:883–925.
- Sekido R, Lovell-Badge R. Genetic control of testis development. *Sex Dev* 2013; **7**:21–32.
- Shaw G, Renfree MB, Leihy MW, Shackleton CH, Roitman E, Wilson JD. Prostate formation in a marsupial is mediated by the testicular androgen 5 alpha-androstane-3 alpha,17 beta-diol. *Proc Natl Acad Sci USA* 2000; **97**:12256–12259.
- Shima Y, Miyabayashi K, Haraguchi S, Arakiwa T, Otake H, Baba TT, Matsuzaki S, Shishido Y, Ariyama H, Tachibana T et al. Contribution of Leydig and Sertoli cells to testosterone production in mouse fetal testis. *Mol Endocrinol* 2013; **27**:63–73.
- Skinner MK, Moses HL. Transforming growth factor β gene expression and action in the seminiferous tubuli: peritubular cell-Sertoli cell interaction. *Mol Endocrinol* 1989; **3**:625–634.
- Skinner MK, Takacs K, Coffey RJ. 1989 Transforming growth factor- α gene expression and action in the seminiferous tubule: peritubular cell-Sertoli cell interactions. *Endocrinology* 1989; **124**:845–854.
- Spade DJ, Hall SJ, Saffarini CM, Huse SM, McDonnell EV, Boekelheide K. Differential response to abiraterone acetate and di-n-butyl phthalate in an androgen-sensitive human fetal testis xenograft bioassay. *Toxicol Sci* 2014; **138**:148–160.
- Sriraman V, Rao VS, Sairam MR, Rao AJ. 2000 Effect of deprivation of LH on Leydig cell proliferation: involvement of PCNA, cyclin D3 and IGF-I. *Mol Cell Endocrinol* 2000; **162**:113–120.
- Sriraman V, Nui E, Matias JR, Donahoe PK, MacLaughlin DT, Hardy MP, Lee MM. Müllerian inhibiting substance inhibits testosterone synthesis in adult rats. *J Androl* 2001; **22**:750–758.
- Stanley E, Lin C-Y, Jin S, Liu J, Sottas CM, Ge R, Zirkin BR, Chen H. Identification, proliferation, and differentiation of adult Leydig stem cells. *Endocrinology* 2012; **153**:5001–5010.
- Stocco DM, Wells J, Clark BJ. The effects of hydrogen peroxide on steroidogenesis in mouse Leydig tumour cells. *Endocrinology* 1993; **133**:2827–2832.
- Suarez-Quian CA, Dai MZ, Onoda M, Kriss RM, Dym M. Epidermal growth factor receptor localization in the rat and monkey testis. *Biol Reprod* 1989; **41**:921–932.
- Svechnikov K, Izzo G, Landreh L, Weisser J, Söder O. Endocrine disruptors and Leydig cell function. *J Biomed Biotechnol* 2010. pii: 684504. doi: 10.1155/2010/684504.
- Svingen T, Koopman P. Building the mammalian testis: origins, differentiation, and assembly of the component cell populations. *Genes Dev* 2013; **27**:2409–2426.
- Swerdlow RS, Heber D. Endocrine control of testicular function from birth to puberty. In: Martini L (ed). *Comprehensive Endocrinology. The Testis*. New York: Raven Press, 1981, 107–126.
- Takao T, Mitchell WM, Tracey DE, de Souza EB. Identification of interleukin-1 receptors in mouse testis. *Endocrinology* 1990; **127**:251–258.
- Tang H, Brennan J, Karl J, Hamada Y, Raetzman L, Capel B. Notch signalling maintains Leydig progenitor cells in the mouse testis. *Development* 2008; **135**:3745–3753.
- Tapanainen J, Kellokumpu-Lehtinen P, Pelliniemi L, Huhtaniemi I. Age-related changes in endogenous steroids of human fetal testis during early and midpregnancy. *J Clin Endocrinol Metab* 1981; **52**:98–102.
- Tapanainen J, Kuopio T, Pelliniemi LJ, Huhtaniemi I. 1984 Rat testicular endogenous steroids and number of Leydig cells between the fetal period and sexual maturity. *Biol Reprod* 1984; **31**:1027–1035.
- Teerds KJ, Dorrington JH. Localization of transforming growth factor β 1 and β 2 during testicular development in the rat. *Biol Reprod* 1993; **48**:40–45.
- Teerds KJ, Rijntjes E. Dynamics of Leydig cell regeneration after EDS. A model for postnatal Leydig cell development. In: Payne AH, Hardy MP (eds). *The Leydig Cell in Health and Disease*. Totowa, New Jersey: Humana Press, Inc., 2007, 91–116.
- Teerds KJ, de Rooij DG, Rommerts FFG, Wensing CJG. The regulation of the proliferation and differentiation of rat Leydig cells after EDS administration or daily hCG treatment. *J Androl* 1988; **9**:343–351.
- Teerds KJ, de Rooij DG, Rommerts FFG, van der Tweel I, Wensing CJG. Turnover time of rat Leydig cells and other interstitial cells in testes of adult rats. *Arch Androl* 1989; **23**:105–111.
- Teerds KJ, Rommerts FFG, Dorrington JH. Immunohistochemical detection of transforming growth factor- α in Leydig cells during the development of the testis. *Mol Cell Endocrinol* 1990; **69**:R1–R6.
- Teerds KJ, de Rooij DG, de Jong FH, van Haaster LH. Development of the adult-type Leydig cell population in the rat is affected by neonatal thyroid hormone levels. *Biol Reprod* 1998; **59**:344–350.
- Teerds KJ, Rijntjes E, Veldhuizen-Tsoerkan MB, Rommerts FFG, de Boer-Brouwer M. The development of rat Leydig cell progenitors *in vitro*: how essential is lutinising hormone? *J Endocrinol* 2007; **194**:579–593.
- Themmen APN, Huhtaniemi IT. Mutations of gonadotropins and gonadotropin receptors: elucidating the physiology and pathophysiology of pituitary-gonadal function. *Endocr Rev* 2000; **21**:551–583.
- Valdes-Socin H, Salvi R, Daly AF, Gaillard RC, Quatresooz P, Tebeu PM, Pralong FP, Beckers H. Hypogonadism in a patient with a mutation in the luteinizing hormone beta-subunit gene. *N Engl J Med* 2004; **351**:2619–2625.

- Van den Driesche S, Walker M, McKinnell C, Scott HM, Eddie SL, Mitchell RT, Seckl JR, Drake AJ, Smith LB, Anderson RA *et al.* Proposed role for COUP-TFII in regulating fetal Leydig cell steroidogenesis, perturbation of which leads to masculinization disorders in rodents. *PLoS One* 2012;**7**:e37064.
- Varjosalo M, Taipale J. Hedgehog: functions and mechanisms. *Genes Dev* 2008;**22**:2454–2472.
- Veldhuis JD, Liu PY, Keenan DM, Takahashi PY. 2012 Older men exhibit reduced efficacy of and heightened potency downregulation by intravenous pulses of recombinant human LH: a study in 92 healthy men. *Am J Physiol Endocrinol Metab* 2012;**302**:E117–E122.
- Verhagen I, Ramaswamy S, Teerds KJ, Keijer J, Plant TM. Time course and role of luteinizing hormone and follicle-stimulating hormone in the expansion of the Leydig cell population at the time of puberty in the rhesus monkey (macaca mulatta). *Andrology* 2014;**2**:924–930.
- Viger RS, Robaire B. Steady state steroid 5 α -reductase messenger ribonucleic acid levels and immunocytochemical localization of the type I protein in the rat testis during postnatal development. *Endocrinology* 1995;**136**:5409–5415.
- vom Saal FS, Hughes C. An extensive new literature concerning low-dose effects of bisphenol A shows the need for a new risk assessment. *Environ Health Perspect* 2005;**113**:926–933.
- Vreeburg JTM, Ooms MP, Rommerts FFG, Teerds KJ. Functional properties of developing rat Leydig cells after treatment with ethylene dimethanesulphonate (EDS). *J Reprod Fertil* 1988;**84**:63–69.
- Wang G, Hardy MP. Development of Leydig cells in the insulin-like growth factor-I (IGF-I) knockout mouse: effects of IGF-I replacement and gonadotropic stimulation. *Biol Reprod* 2004;**70**:632–639.
- Wang X-J, Stocco DM. The decline in testosterone biosynthesis during male aging: a consequence of multiple alterations. *Mol Cell Endocrinol* 2005;**238**:1–7.
- Wu X, Arumugam R, Baker SP, Lee MM. Pubertal and adult Leydig cell function in Müllerian inhibiting substance-deficient mice. *Endocrinology* 2005;**146**:589–595.
- Wu X, Wan S, Lee MM. Key factors in the regulation of fetal and postnatal Leydig cell development. *J Cell Physiol* 2007;**213**:429–433.
- Wu FC, Tajar A, Pye SR, Silman AJ, Finn JD, O'Neill TW, Bartfai G, Casanueva F, Forti G, Giwercman A *et al.* Hypothalamic-pituitary-testicular axis disruptions in older men are differentially linked to age and modifiable risk factors: the European Male Ageing Study. *J Clin Endocrinol Metab* 2008;**93**:2737–2745.
- Wu FCW, Tajar A, Beynon JM, Pye SR, Silman AJ, Finn JD, O'Neill TW, Bartfai G, Casanueva FF, Forti G *et al.* Identification of late-onset hypogonadism in middle-aged and elderly men. *N Engl J Med* 2010;**363**:123–135.
- Xu Z, Yu S, Hsu CH, Eguchi J, Rosen ED. The orphan nuclear receptor chicken ovalbumin upstream promoter-transcription factor II is a critical regulator of adipogenesis. *Proc Natl Acad Sci USA* 2008;**105**:2421–2426.
- Yamamura K, Doi M, Hayashi H, Ota T, Murai I, Hotta Y, Komatsu R, Okamura H. 2014 Immunolocalization of murine type VI 3 β -hydroxysteroid dehydrogenase in the adrenal gland, testis, skin, and placenta. *Mol Cell Endocrinol* 2014;**382**:131–138.
- Yao HH, Whoriskey W, Capel B. 2002 Desert Hedgehog/Patched 1 signaling specifies fetal Leydig cell fate in testis organogenesis. *Genes Dev* 2002;**16**:1433–1440.
- Zaidi SK, Shen W-J, Azhar S. Impact of aging on steroid hormone biosynthesis and secretion. *Open Longev Sci* 2012;**6**:1–30.
- Zhang FP, Hamalainen T, Kaipia A, Pakarinen P, Huhtaniemi I. Ontogeny of luteinizing hormone receptor gene expression in the rat testis. *Endocrinology* 1994;**134**:2206–2213.
- Zhang FP, Poutanen M, Wilbertz J, Huhtaniemi I. Normal prenatal but arrested postnatal sexual development of luteinizing hormone receptor knockout (LuRKO) mice. *Mol Endocrinol* 2001;**15**:172–183.
- Zhang FP, Pakarainen T, Zhu F, Poutanen M, Huhtaniemi I. Molecular characterization of postnatal development of testicular steroidogenesis in luteinizing hormone receptor knockout mice. *Endocrinology* 2004;**145**:1453–1463.
- Zhang L, Wang H, Yang Y, Liu H, Zhang Q, Xiang Q, Ge R, Su Z, Huang Y. NGF induces adult stem Leydig cells to proliferate and differentiate during Leydig cell regeneration. *Biochem Biophys Res Commun* 2013;**436**:300–305.
- Zhou Y, Xu BC, Maheshwatri HG, Li H, Reed M, Lozykowski M, Okada S, Catalog L, Coschigamo K, Wagner TE *et al.* A mammalian model for Laron syndrome produced by targeted disruption of the mouse growth hormone receptor/binding protein gene (the Laron mouse). *Proc Natl Acad Sci USA* 1997;**94**:13215–13220.
- Zimmermann S, Schottler P, Engel W, Adham IM. Mouse Leydig insulin-like (Ley I-L) gene: structure and expression during testis and ovary development. *Mol Reprod Dev* 1997;**7**:30–38.
- Zirkin BR, Tenover LJ. Aging and declining testosterone: past, present and hopes for the future. *J Androl* 2012;**33**:1111–1118.
- Zirkin BR, Santulli R, Strandberg JD, Wright VVW, Ewing LL. Testicular steroidogenesis in the aging Brown Norway rat. *J Androl* 1993;**14**:118–123.