Chapter 5
Origin and Differentiation of Androgen-Producing Cells in the Gonads

Sarah J. Potter, Deepti Lava Kumar, and Tony DeFalco

Abstract Sexual reproduction is dependent on the activity of androgenic steroid hormones to promote gonadal development and gametogenesis. Leydig cells of the testis and theca cells of the ovary are critical cell types in the gonadal interstitium that carry out steroidogenesis and provide key androgens for reproductive organ function. In this chapter, we will discuss important aspects of interstitial androgenic cell development in the gonad, including: the potential cellular origins of interstitial steroidogenic cells and their progenitors; the molecular mechanisms involved in Leydig cell specification and differentiation (including Sertoli-cell-derived signaling pathways and Leydig-cell-related transcription factors and nuclear receptors); the interactions of Leydig cells with other cell types in the adult testis, such as Sertoli cells, germ cells, peritubular myoid cells, macrophages, and vascular endothelial cells; the process of steroidogenesis and its systemic regulation; and a brief discussion of the development of theca cells in the ovary relative to Leydig cells in the testis. Finally, we will describe the dynamics of steroidogenic cells in seasonal breeders and highlight unique aspects of steroidogenesis in diverse vertebrate species. Understanding the cellular origins of interstitial steroidogenic cells and the pathways directing their specification and differentiation has implications for the study of multiple aspects of development and will help us gain insights into the etiology of reproductive system birth defects and infertility.

5.1 Origins of Androgen-Producing Cells (Cell Migration and Progenitors)

Androgen-producing cells differentiate in the interstitium of the mammalian gonad, outside of the germ-cell-containing compartments of the testis (testis cords/seminiferous tubules) and ovary (follicles). While steroidogenic cells of the male gonad,

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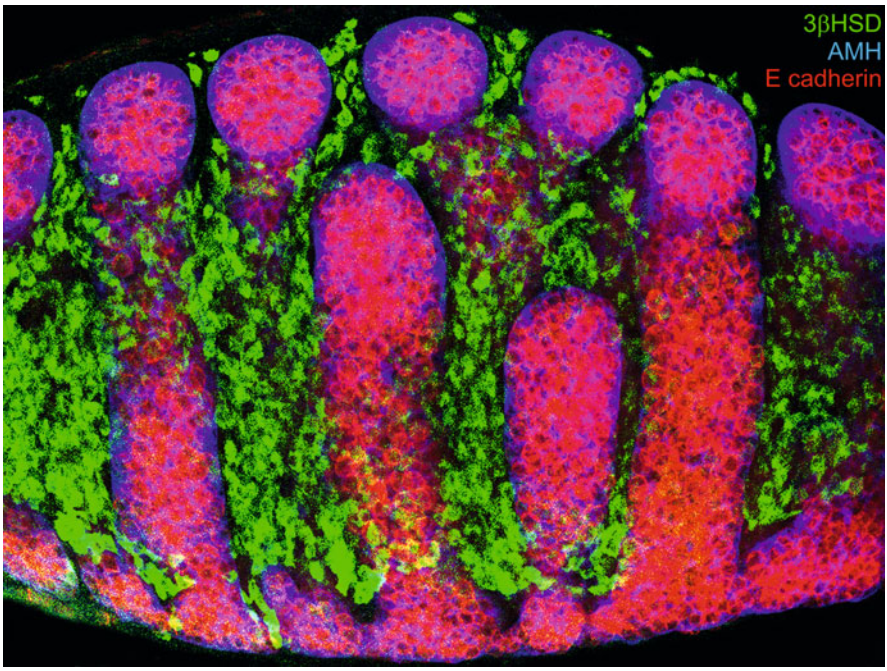


Fig. 5.1 Leydig cells differentiate in the interstitium of the fetal testis. Confocal microscopy image of a 13.5 *dpc* fetal mouse XY gonad, showing the development of two main testicular compartments: the testis cords, which contain Sertoli cells (marked in *blue* with anti-AMH antibody) and germ cells (marked in *red* with anti-Cadherin1/E-cadherin antibody), and the interstitium, which contains differentiated steroidogenic Leydig cells (marked in *green* with anti-HSD3B1/3β-HSD antibody)

termed Leydig cells (after the German anatomist Franz Leydig who first described them), arise during fetal development (Fig. 5.1), theca cells, one of the steroidogenic cell populations of the ovary which produces androgens, do not differentiate until perinatal stages when follicular development begins (Barsoum and Yao 2010; Magoffin 2005; Mannan and O'Shaughnessy 1991).

Fetal Leydig cells (FLCs), the steroidogenic population in the developing testis, arise 24 h after Sertoli cell specification at approximately 12.5 *dpc* (days *post coitum*) in the mouse fetal gonad (Barsoum and Yao 2010). The number of FLCs increases dramatically from 12.5 to 18.5 *dpc* (Barsoum and Yao 2010), after which FLC number decreases over the first week of postnatal life. FLCs are gradually replaced by adult Leydig cells (ALCs), which arise anew postnatally (i.e., not directly derived from differentiated FLCs; this is discussed in greater detail later in this chapter). As differentiated FLCs and ALCs are mitotically inactive (Byskov 1986; Migrenne et al. 2001; Orth 1982), the expansion of both cell types depends on the differentiation of progenitor cells in the interstitium. The origin of FLCs has not yet been identified definitively and has been a subject of debate for many years. Nevertheless, multiple potential sources have been speculated for the origin of FLCs (Fig. 5.2). These include neural crest cells, coelomic epithelium,

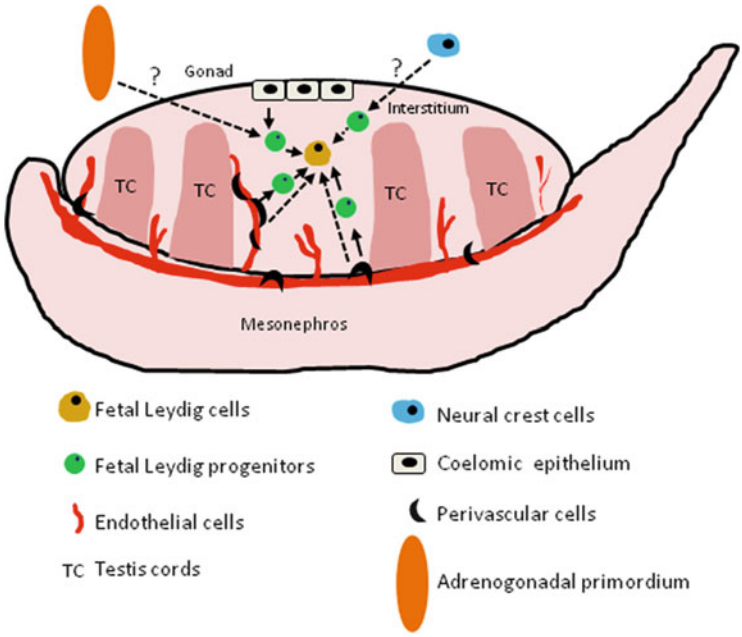


Fig. 5.2 Potential origins of fetal Leydig cells. Schematic of a fetal mouse XY gonad, showing potential cellular sources that give rise to fetal Leydig cells and their progenitors. *Arrows* indicate cellular sources that have been suggested in the literature (neural crest cells, coelomic epithelium, mesonephros, adrenogonadal primordium, and perivascular cells), while *question marks* indicate cellular sources that are still untested (adrenogonadal primordium) or are unsupported or inconsistent with data in the field (neural crest cells). It is still unclear for some cell populations whether they initially give rise to progenitors or if they directly differentiate into mature Leydig cells. The events depicted likely do not occur simultaneously, but can occur over a span of 48 h in the mouse (11.5–13.5 *dpc*)

mesonephros, adrenogonadal primordium, and perivascular cells. While it is assumed that female androgen-producing cells arise from similar progenitors as their male counterparts, not as much is known about the fetal origin of theca cells; we have discussed theca cell origin relative to Leydig cells in greater detail in Sect. 5.6. Other cell types in the gonad, such as Sertoli cells, granulosa cells, and luteal cells, also produce steroid hormones and other factors involved in steroid hormone regulation; however, we will focus on the interstitial androgen-producing cells, as the other cell types will be discussed in greater detail in other chapters in this volume.

Neural crest cells Neural crest cells (NCCs) arise from a group of precursor cells located between the neural plate and the epidermis in the developing embryo and give rise to different cell types of the body. Studies have shown that NCCs can contribute to neuroendocrine populations of the developing embryo, including the steroidogenic cells of the adrenal medulla and neuroendocrine cells in the pancreas and foregut (Huber 2006). Despite some contradictory findings with respect to the

pancreas and the foregut, the hypothesis that NCCs are putative precursors for neuroendocrine cells has persisted (Andrew and Kramer 1979; Andrew et al. 1998; Kirchgessner et al. 1992; Pictet et al. 1976). Given that the adrenal gland and the gonad share a common origin, i.e., arising from the same primordium (Griswold and Behringer 2009), along with studies showing that NCCs might be precursors for neuroendocrine populations (Griswold and Behringer 2009), it was reasonable to hypothesize that FLCs arise from NCCs; additionally, it was shown that Sertoli cells and NCCs have common DNA regulatory elements for testis-expressed genes such as *Gata4* and *Sry* (Boyer et al. 2006; Pilon et al. 2008). Studies have also shown that fetal and adult Leydig cells express a number of neural markers such as Nestin, Neural cell adhesion molecule (NCAM, official name NCAM1), S-100, and Neurofilament protein 200 (official name Neurofilament, heavy polypeptide/NEFH) (Davidoff et al. 2002; Lobo et al. 2004; Middendorff et al. 1993; Mayerhofer et al. 1996). These neuroendocrine characteristics of the FLCs led to the idea that one of the potential sources of FLC origin could be NCCs. To determine if NCCs give rise to FLCs, Brennan and colleagues lineage traced NCCs using a Cre-responsive *lacZ* reporter strain driven by two distinct NCC-specific Cre lines (under the control of either the *P0* or *Wnt1* promoter), but did not find significant evidence for the contribution of NCCs toward Leydig cell lineages (Brennan et al. 2003). These findings suggested that FLCs do not originate from the neural crest cell lineage and instead acquire their neuroendocrine properties later on during development of the testis.

Coelomic epithelium Another potential source for the origin of FLCs is the coelomic epithelium of the gonad. Karl and Capel used a lipophilic dye (DiI) to label the cells of the coelomic epithelium and tracked them for 12–24 h in *ex vivo* organ explant cultures (Karl and Capel 1998). The migration of labeled cells into the testicular compartment and their subsequent differentiation were dependent upon the developmental stage at which the gonad was exposed to the dye. When the dye was injected at 11.2–11.4 *dpc* (during 15–17 tail-somite stages), the labeled cells were able to migrate into the gonad and were found in the testis cords (suggestive of a Sertoli cell fate) as well as in the interstitium (suggestive of FLC fate). In later stages of development, i.e., at 11.5 *dpc* or later (18–20 tail-somite stages), labeled cells were excluded from the testis cords and were restricted to the interstitium. Whether these labeled cells directly gave rise to FLCs was not specifically tested. However, DeFalco et al. (2011) performed similar dye-labeling experiments using a MitoTracker dye and showed that a subset of later-stage (11.5 *dpc* or later) labeled cells expressed the Leydig-cell-specific enzyme 3β-HSD (official name HSD3B1), demonstrating definitively that the coelomic epithelium is one of the cellular origins of FLCs. Further studies are required to establish the specific roles of the progeny of the coelomic epithelium at different stages with regard to FLC development.

Mesonephros The mesonephros, the organ that borders the gonad, is another tissue that is thought to contain precursors of FLCs. The mesonephros gives rise to the extra-gonadal ducts of the reproductive tract, such as the epididymis and oviduct in males and females, respectively. In mice, shortly after sex determination at around

12.5 *dpc*, one of the dramatic changes that occur in the male gonad is migration of cells from the mesonephros. This migration is induced by the male gonad (the sex of the mesonephros does not matter) and is dependent on *Sry* function (Capel et al. 1999; Martineau et al. 1997). Studies using gonad/mesonephros recombination organ cultures (male wild-type gonads cultured on top of a mesonephros carrying a ubiquitously expressed GFP or *lacZ* reporter under the *β-actin* or *ROSA26* promoter, respectively) demonstrated that cells from the adjacent mesonephros contribute to the gonadal population. GFP- and *lacZ*-positive cells from the mesonephros were shown to migrate into the gonad, but most of these cells were characterized as endothelial cells (Combes et al. 2009; Cool et al. 2008; Martineau et al. 1997). Additionally, using *kinase insert domain protein receptor* (*Kdr*)-*lacZ* mice (mice with *lacZ* expression driven by the promoter of *Kdr*, the gene encoding VEGFR2), it was shown that *Kdr-lacZ*-expressing endothelial cells from the mesonephros migrated in the XY gonad at 11.5 *dpc* (Bott et al. 2010). This migratory event occurs specifically in the male gonad and is essential for normal testis cord formation (Tilmann and Capel 1999). Further research is required to determine definitively if any non-endothelial mesonephric cells that migrate into the gonad originate from the mesenchyme or epithelia of the mesonephros, and if any cells that may migrate during earlier gonad development (i.e., prior to the stages when gonad–mesonephros recombination cultures can be set up) contribute to the FLC population.

Steroidogenic factor-1 (SF-1)-positive progenitors in the adrenogonadal primordium Studies have shown that 11.5 *dpc* (a stage prior to the migration of cells from the mesonephros) male gonads cultured in the absence of the mesonephros failed to develop testis cords, but did contain a subpopulation of cells that have FLC characteristics (Merchant-Larios et al. 1993). This indicates that the precursors that give rise to the FLC population are present in the gonad prior to the migratory event that takes place from the adjacent mesonephros. Both Sertoli cells and Leydig cells express SF-1 (Steroidogenic factor 1; official name is nuclear receptor subfamily 5, group A, member 1 [NR5A1]), and hence, it has been suggested that they arise from progenitors that express SF-1 (Luo et al. 1994, 1995). SF-1 expression begins early on in adrenogonadal development and SF-1-positive cells are known to contribute to steroidogenic cells in the adrenal cortex. Whether these SF-1-positive cells are the FLC precursors present in the adrenogonadal primordium or whether the precursors arise as a separate population and subsequently initiate SF-1 expression independently is not known and warrants further testing.

Perivascular cells and pericytes DeFalco et al. (2011) performed a detailed analysis of the cells that form the interstitial compartment of the developing mouse gonad. They found that the interstitium is comprised of a heterogeneous population of cells, some of which are closely associated with the vasculature during organ formation. Using immunofluorescence, the authors identified expression of the Maf family of basic leucine zipper transcription factors, specifically MAFB and C-MAF (official name MAF), within the interstitium. These factors are the mammalian orthologs of the *Drosophila* gene *traffic jam* known to regulate cell adhesion

interactions during morphogenesis of the fly gonad (Li et al. 2003) and were examined for their potential roles in mammalian gonad development; MAFB and C-MAF were found to be early markers of the interstitial lineage. MAFB was dynamically expressed in the gonad with expression along the gonad–mesonephros border in 11.5 *dpc* embryos and in some interstitial cells in both male and female gonads. Additionally, some scattered cells at 11.5 *dpc* also showed expression of MAFB in the gonadal surface domain just beneath the coelomic epithelium only in the male gonad; the expression of MAFB was mutually exclusive with Sertoli cell markers, suggestive of an early interstitial fate. By 12.5 *dpc*, expression expanded to most of the somatic cells in the male interstitium, i.e., outside the testis cords. By 13.5 *dpc* MAFB and C-MAF were expressed in most interstitial cells. Later on in fetal life (by 14.5 *dpc*), *Mafb*-GFP knock-in reporter expression was observed specifically in the Leydig cell lineage. C-MAF expression was also observed along the gonad–mesonephros border and also within interstitial cells, although the onset of its expression was slightly delayed relative to MAFB; later expression of C-MAF was restricted to non-Leydig interstitial cells (DeFalco et al. 2011). Subsequent work revealed that some of the C-MAF-positive cells are gonadal macrophages (DeFalco et al. 2014). The MAFB/C-MAF double-positive interstitial cells likely represent Leydig progenitors during initial testis formation, and these two factors subsequently segregate into unique lineages, similar to MAFA and MAFB during alpha and beta cell specification in the pancreas (Nishimura et al. 2009).

Additionally, to determine if MAFB/C-MAF-positive perivascular cells along the gonad–mesonephric border migrate into the gonad and contribute to the interstitial population, a modified GFP recombination assay (see above) was performed by DeFalco and colleagues (DeFalco et al. 2011). Usually vascular sprouts and associated perivascular cells are disrupted during preparation of gonadal and mesonephric tissues in recombination assays, and GFP-positive migratory cells are strictly endothelial cells (Combes et al. 2009). However, in this case, vascular sprouts and associated cells were kept intact in the mesonephric component of the recombination culture. The researchers found that the GFP-positive cells also expressed VCAM1, a marker for vasculature-associated interstitial cells, and these cells migrated into the gonad. They also showed that these GFP-positive interstitial cells were adjacent to GFP-positive endothelial cells, suggesting that these two cell types migrated into the gonad together. Staining with 3β-HSD, a marker for differentiated FLCs, following a 64-h culture (time during which the cells would differentiate into Leydig cells), revealed 3β-HSD expression in GFP-positive cells (DeFalco et al. 2011). Thus, these findings indicated that migrating perivascular cells at the gonad–mesonephros border represent a progenitor population that gives rise to fetal Leydig cells.

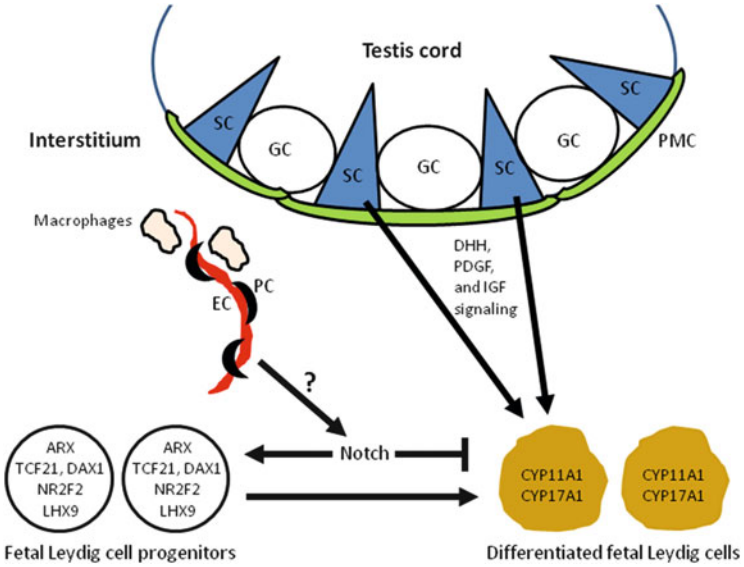


Fig. 5.3 Mechanisms regulating fetal Leydig cell differentiation. Signaling pathways and factors involved in maintaining a balance between fetal Leydig cell progenitors and differentiated fetal Leydig cells in the interstitium of the XY gonad. The *question mark* indicates the unknown relationship between vasculature/perivascular cells and Notch signaling. The transcription factors mentioned in the progenitor cell population are expressed in these cells but do not necessarily promote progenitor identity, as many are responsible for promoting differentiation. Abbreviations are as follows: *EC* endothelial cells, *GC* germ cells, *PC* pericytes, *PMC* peritubular myoid cells, *SC* Sertoli cells

5.2 Molecular Mechanisms of FLC Differentiation

5.2.1 Signaling Pathways Regulating FLC Development

Regardless of their origin, FLCs lack expression of *Sry* or *Sox9* and hence their male-specific differentiation likely depends on molecular cues from the neighboring Sertoli cells (Barsoum and Yao 2010). For example, Sertoli-cell-derived signaling molecules such as Desert hedgehog (DHH) and Platelet-derived growth factor alpha (PDGFA) have been shown to positively regulate FLC differentiation (Brennan et al. 2003; Yao et al. 2002). Balance between differentiation-promoting and differentiation-inhibiting factors regulates maintenance and differentiation of the Leydig cell population in the fetal gonad (Fig. 5.3).

Hedgehog signaling In the XY gonad, *desert hedgehog* (*Dhh*) mRNA is expressed in Sertoli cells beginning at 11.5 *dpc* and the gene encoding its corresponding receptor, *patched homolog 1* (*Ptch1*), is expressed in the interstitium of only XY gonads at around 12.5 *dpc* (Yao et al. 2002). XY gonads lacking *Dhh* show defects in the differentiation of FLCs and have disruptions in testis cord formation

(Yao et al. 2002). In prepubertal and adult stages, *Dhh*-null mice show spermatogenetic defects and lack adult Leydig cells (Clark et al. 2000). Humans with mutation in the *DHH* gene show pseudohermaphroditism and gonadal dysgenesis, a phenotype similar to *Dhh*-null mice (Canto et al. 2004; Umehara et al. 2000). *Dhh* is thought to act by regulating the expression of *Sf-1* and *cytochrome P450, family 11, subfamily a, polypeptide 1* (*Cyp11a1*, also called *side chain cleavage* [*Scc*], a steroidogenic enzyme required for androgen synthesis) genes (Barsoum and Yao 2010). These data suggest that DHH secreted from Sertoli cells signals to the interstitium, which expresses the PTCH1 receptor, to determine FLC cell fate (Yao et al. 2002).

Platelet-derived growth factor (PDGF) signaling The PDGFA ligand is secreted from Sertoli cells and is thought to signal to its receptor PDGFRA, which is expressed throughout the mesenchyme of the XY interstitium (fewer cells express PDGFRA in the XX interstitium) (Brennan et al. 2003). While both PDGFRA and PDGFRB receptors are expressed in the interstitium, only *Pdgfra* function is uniquely required for testis differentiation: analyses of *Pdgfrb*-mutant embryos revealed no gross defects in the early fetal testis (Brennan et al. 2003). The *Pdgfra*-null XY gonad showed a disruption in the formation of testis-specific vasculature and patterning of the testis cords; mesonephric cell migration and proliferation of interstitial FLC precursors were also severely reduced (Brennan et al. 2003). This indicates that PDGFA-PDGFRA signaling plays an important role in testis organogenesis and Leydig cell differentiation (Brennan et al. 2003). These studies strongly suggest that a network of signaling from the Sertoli cells to the mesenchymal cells of the interstitium is responsible for regulating the process of FLC differentiation.

Insulin-like growth factor (IGF) signaling In addition to Hedgehog and PDGF signaling pathways, Insulin-like growth factor (IGF) signaling from the Sertoli cells is also known to positively regulate FLC differentiation (Baker et al. 1996). *Insulin-like growth factor 1* (*Igf1*)-mutant mice are infertile dwarfs with dramatically reduced testosterone levels, causing a failure of masculinization and subsequent infertility (Baker et al. 1996). Further analyses of mutant Leydig cells showed that there is a developmental delay, pointing toward an essential role of *Igf1* in Leydig cell differentiation (Baker et al. 1996), although it is possible that Leydig cell differentiation is altered due to delays or defects in testis cord development in IGF pathway mutants (Nef et al. 2003; Pitetti et al. 2013).

Notch signaling While Sertoli-cell-derived DHH and PDGFA positively regulate FLC differentiation, Notch signaling was shown to negatively regulate FLC differentiation (Tang et al. 2008). Inhibition of Notch signaling in the fetal testis using DAPT (*N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester), a gamma-secretase inhibitor, or disruption of the Notch target gene *hairy and enhancer of split 1* (*Hes1*) resulted in an increase in the number of differentiated FLCs (Tang et al. 2008). Conversely, constitutive activation of Notch in the somatic gonad using a *Sf-1*-Cre-driven *Rosa*-Notch1-intracellular-domain transgene led to a decrease in FLC number (Tang et al. 2008). Thus, Notch signaling in the fetal

gonad promotes the maintenance of the Leydig progenitor cell population by restricting Leydig cell differentiation within the interstitial compartment. The exact cell types engaging in Notch signaling have not been clearly defined; however, a recent study showed that the activity of *Jag1* (a gene encoding one of the Notch ligands) is required in the interstitium and perivascular cells for a proper balance between progenitors and differentiated cells, and JAG1 signaling may act through the receptor NOTCH2 (DeFalco et al. 2013).

5.2.2 Transcription Factors and Nuclear Receptors Involved in FLC Differentiation

Apart from paracrine factors, studies have shown that several transcription factors and nuclear receptors play a role in regulating the differentiation of FLCs. These factors likely act downstream of key signaling pathways (described above) cell autonomously to regulate key aspects of steroidogenic development within interstitial cells.

Transcription factor 21 (TCF21) Transcription factor 21 (TCF21) (also called Pod1/Capsulin or Epicardin), a basic helix-loop-helix transcription factor, negatively regulates FLC differentiation (Cui et al. 2004). While *Tcf21*-mutant mice die at birth due to respiratory failure caused by the absence of alveoli, the authors described feminization of the external genitalia in XY gonads from *Tcf21*-mutant mice. The effect of *Tcf21* mutation on gonad development was revealed to be an ectopic expression of SF-1; this upregulation of SF-1 resulted in more progenitor cells committing to a steroidogenic cell fate, resulting in abnormal steroidogenesis (Cui et al. 2004).

Aristaless-related homeobox (ARX) ARX is another transcription factor that plays a role in FLC differentiation (Kitamura et al. 2002; Miyabayashi et al. 2013). *Arx*-mutant testes show defective Leydig cell differentiation with a decrease in FLC number throughout fetal life. Since differentiated Leydig cells do not proliferate, this decrease in Leydig cell number is attributed to defects in the Leydig cell progenitor population. Miyabayashi et al. (2013) showed that the progenitor population that gives rise to differentiated FLCs is ARX positive. The authors' general conclusion was that the ARX transcription factor positively regulates fetal Leydig cell differentiation (Miyabayashi et al. 2013).

LIM homeobox protein 9 (LHX9) LHX9 belongs to the LIM homeobox gene family of transcription factors (Birk et al. 2000). It is known to play an important role in gonadal primordium development, as mice lacking *Lhx9* show gonadal agenesis (lack of gonads), lack male accessory sex organs, and are infertile (Birk et al. 2000). In early-stage embryos (11.5 *dpc*), *Lhx9* is highly expressed in the somatic progenitors of the coelomic epithelium and the subjacent mesenchyme. Tang et al. (2008) reported an increase in the number of LHX9-positive spindle-

shaped progenitor cells and a concomitant decrease in the number of differentiated Leydig cells following constitutive activation of Notch signaling in the male fetal gonad. As spindle-shaped cells have been identified as putative adult Leydig stem cells (Davidoff et al. 2004), it is likely that these spindle-shaped LHX9-positive cells represent a progenitor pool that gives rise to differentiated FLCs. Whether these LHX9-positive progenitor cells directly give rise to differentiated FLCs is not known. Nevertheless, the authors concluded that constitutive activation of Notch signaling maintains the LHX9 progenitor population and likely prevents their differentiation into Leydig cells (Tang et al. 2008).

Nuclear receptor subfamily 0, group B, member 1 (NR0B1) Nuclear receptor subfamily 0, group B, member 1 (NR0B1), also called DAX1, is a nuclear receptor that plays an important role in testis cord organization during gonadal differentiation (Meeks et al. 2003). Gonads lacking *Dax1* show a host of testis defects, such as abnormal organization of Sertoli cells, lack of basal laminae in the testis, and disrupted peritubular myoid cell development. Both the number and organization of immature Leydig cells, as shown by the expression of CYP11A1 (a steroidogenic enzyme required for androgen synthesis), were affected. Immunohistochemical staining with SF-1 revealed that Leydig cells were restricted to the coelomic surface in *Dax1*-mutant mice (Meeks et al. 2003).

Nuclear receptor subfamily 2, group F, member 2 (NR2F2) Nuclear receptor subfamily 2, group F, member 2 (NR2F2), also called COUP-TFII, is a member of the nuclear receptor family of proteins and was also shown to play an essential role in differentiation of both fetal and adult Leydig cells (Qin et al. 2008). *Nr2f2* is highly expressed in the mesenchyme and is absolutely essential for development, as embryos lacking a functional copy of *Nr2f2* die at 10.5 *dpc* due to angiogenic and cardiovascular defects (Pereira et al. 1999; Tsai and Tsai 1997). In rats, between 15.5 and 21.5 *dpc* there is a threefold increase in FLC number, cytoplasmic volume per Leydig cell, and intratesticular testosterone levels, along with a decrease in the Leydig cell population expressing NR2F2. Exposing fetal rats to dibutyl phthalate (DBP), a drug that induces masculinization disorders, prevented this decrease in NR2F2-expressing Leydig cells and also prevented a normal age-dependent change in Leydig cell cytoplasmic volume, nuclear volume, and ITT levels, but did not have any effect on FLC number at any age. Furthermore, exposure to DBP downregulated the expression of SF-1-regulated steroidogenic genes such as *StAR*, *Cyp11a1*, and *Cyp17a1* (with binding sites for both SF-1 and NR2F2 in their promoter regions), but not *3β-HSD* (with binding sites for only SF-1 in its promoter region). The authors concluded that repression of NR2F2 may be an important mechanism by which fetal Leydig cells regulate the production of testosterone to ultimately drive masculinization (van den Driesche et al. 2012). To determine the role of *Nr2f2* in postnatal stages, conditional ablation of the *Nr2f2* gene was achieved using a ubiquitously expressed tamoxifen-inducible Cre line (Qin et al. 2008). Loss of *Nr2f2* during prepubertal stages following injection of tamoxifen at postnatal day 14 resulted in defective testosterone biosynthesis that led

to arrest of spermatogenesis at the round spermatid stage, ultimately leading to infertility (Qin et al. 2008).

5.3 Adult Leydig Cell Sources and Differentiation

5.3.1 Sources of Adult Leydig Cells

Unlike most cell types, Leydig cells in the adult testis do not directly arise from Leydig cells in the fetal testis; instead they differentiate anew in postnatal life as fetal cells disappear. It has been suggested that both FLCs and ALCs originate from stem cell precursors (Benton et al. 1995; Ge et al. 2006; Hardy et al. 1989, 1990), but whether FLCs and ALCs arise from the same stem cell progenitor has not been fully resolved. Additionally, the source of the stem Leydig cell (SLC) that gives rise to the ALC is unclear; possibilities include perivascular smooth muscle and pericytes (Davidoff et al. 2004) or peritubular mesenchyme cells (Haider et al. 1995).

Davidoff et al. (2004, 2009) reported that vascular smooth muscle cells and pericytes present in the testis represent the progenitor population that gives rise to adult Leydig cells (ALCs). The putative Leydig cell progenitors (i.e., vascular smooth muscle cells and pericytes) expressed the intermediate filament protein Nestin, also known to be expressed in neural stem cells and progenitors (Davidoff et al. 2004, 2009). Furthermore, to determine if these Nestin-positive progenitors give rise to the ALC population, the authors injected ethane dimethane sulfonate (EDS) into adult rats. EDS is a cytotoxic compound that specifically eliminates the existing Leydig cell population temporarily; however, Leydig cells start regenerating within 2 weeks and are completely recovered by 1 month. Fourteen days following EDS treatment, Nestin-positive cells reappeared as clusters near the vicinity of the blood vessels (Nestin was used as a marker for Leydig progenitors). These Nestin-positive cells were identified as vascular smooth muscle cells and pericytes. Using Nestin-GFP transgenic mice it was recently demonstrated that Nestin-GFP-positive cells have the ability to undergo self-renewal in vitro and are able to differentiate into Leydig cells (Jiang et al. 2014). Additionally, when these Nestin-positive cells were transplanted into Leydig-cell-depleted mice (following injection of EDS), these cells were able to colonize the interstitium, differentiate into Leydig cells, partially rescue testosterone deficiency, and thereby improve spermatogenesis, as testosterone is known to be critical for completion of meiosis and spermiogenesis in rodents (McLachlan et al. 2002). In all, it was shown that perivascular cells and pericytes can give rise to adult Leydig cells.

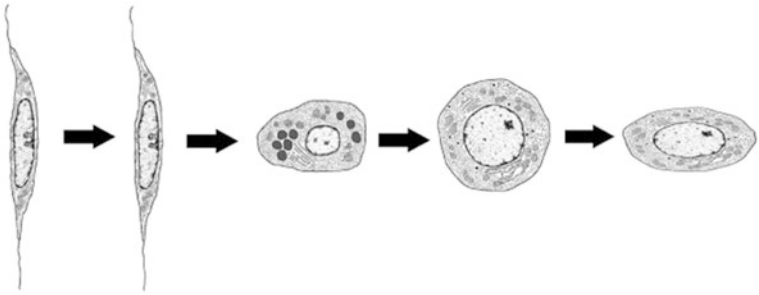
SLCs are capable of differentiation into the Leydig lineage, as SLCs give rise to ALCs when transferred to postnatal mice, resulting in ALC reconstitution (Ge et al. 2006). SLCs that give rise to ALCs express growth factor receptors, such as those for Kit ligand/Stem cell factor (SCF), Leukemia inhibitory factor

(LIF), and Platelet-derived growth factor alpha (PDGFA), which allow SLCs to functionally respond to these mitogens by proliferating (Ge et al. 2006). Decreased androgen receptor (AR) expression and DBP-induced epigenetic changes both influence the SLC population, thus affecting ALC differentiation (Kilcoyne et al. 2014; O'Shaughnessy et al. 2002). Factors, such as SF-1, a Leydig-cell-related transcription factor, and DHH, a Sertoli-cell-released factor, both have roles in FLC differentiation and influence ALCs, suggesting that the presence of FLCs or their related intrinsic or extrinsic factors may be involved in ALC development (Park et al. 2007). Yang et al. (2015) showed that the development of ALCs from mouse embryonic stem (mES) cells requires SF-1, supporting the role of intrinsic FLC-related transcription factors in the development of ALCs. Alternatively, Kilcoyne et al. (2014) via lineage tracing in rats determined that NR2F2-positive cells, the putative progenitor cell population for ALCs, exist during fetal stages. Therefore, ALC stem cells might be the same progenitor population for both FLCs and ALCs. Furthermore, FLC and ALC populations overlap during the first few weeks of postnatal life in rodents, when FLCs decline and are slowly replaced with ALCs, leaving a small number of FLCs remaining in adulthood (Benton et al. 1995).

5.3.2 *Differentiation of Adult Leydig Cells from Stem Cell Progenitors*

In humans, there is a triphasic progression of Leydig cells: fetal (first trimester), postnatal (2–3 months), and adult stages (puberty), all of which have distinct characteristics (Forest et al. 1973; Prince 1990; Reyes et al. 1974). In humans and pigs, FLCs (discussed in earlier sections) reach their maximum number by the end of fetal development, regressing in both number and size during neonatal periods followed by the propagation of a new subset of Leydig cells. The postnatal population peaks and also regresses by the first wave of spermatogenesis. This is followed by the appearance of a more permanent population that persists throughout adulthood (Prince 1990). In rats, most studies suggest a biphasic pattern, with FLCs peaking and then declining around the time when ALCs arise (Christensen and Gillim 1969). This difference may be due to the timescale of human gestation as compared to rodents.

Differentiation Although several studies have addressed morphological and gene expression differences between FLCs and the progenitor stages of adult Leydig cells (ALC) and others have analyzed SLC and ALC gene expression (Stanley et al. 2011), the differences between the terminally differentiated populations of FLCs and the ALCs have not been fully elucidated. In the rat model, the stem Leydig cells (SLCs) arise around postnatal day 7 and differentiate through various discrete stages resulting in mature ALCs by postnatal days 28–90 (Chen et al. 2010; Ge et al. 2006; Stanley et al. 2011) (Fig. 5.4 depicts the timescale and



CELLULAR ATTRIBUTES	STEM LEYDIG CELL	PROGENITOR LEYDIG CELL	IMMATURE LEYDIG CELL	ADULT LEYDIG CELL	AGED LEYDIG CELL
Mitotic activity	Low	high	Once	Probably none	Probably none
Morphology	Spindle	Spindle	Round with large lipid droplets	Round with small lipid droplets	Round with small lipid droplets
Smooth ER	Low	Low	High	Highest	High
Testosterone Metabolism	None	High	Highest	Low	Low
Testosterone Synthesis	None	Low	Intermediate	High	Intermediate
Age when cell is typical	7 days	21 days	35 days	90 days	20 months
LH Receptor Number	None	Low	Intermediate	High	Intermediate
Androgen Receptor Number	?	High	High	Low	?

Fig. 5.4 Adult Leydig cell development. Diagram describing the characteristics (mitotic activity, morphology, smooth ER, testosterone metabolism, testosterone synthesis, age when cell is typical, LH receptor number, and androgen receptor number) of adult Leydig cell progenitors through differentiated cell populations in postnatal through aged rats. This figure is from Chen et al. (2009), used with permission from Elsevier and *Molecular and Cellular Endocrinology*. Abbreviation is as follows: *ER* endoplasmic reticulum. Refer to Sect. 5.3.2 in text

characteristics of each stage). The SLCs differentiate into progenitor Leydig cells (PLCs), which are characterized by their expression of luteinizing hormone receptor (LHR), 3α-HSD, and 3β-HSD. PLCs appear more spindle shaped and have negligible smooth ER, but still retain the ability to secrete androsterone as compared to SLCs. The PLCs enlarge, become round, and decrease their proliferative capacity, transitioning into immature Leydig cells (ILCs). ILCs are considered steroidogenic, containing abundant amounts of smooth ER and cytoplasmic lipid droplets. ILCs contain high levels of 5α-androstan-3α,17β-diol (3α-diol) (the inactive form of 5α-dihydrotestosterone [DHT]) and undergo a single mitotic division to produce ALCs (Chen et al. 2009). ALCs are large post-mitotic polyhedral cells, which have decreased lipid droplets and decreased levels of 5α-androstan-3α. Although the ALCs themselves do not proliferate (Teerds et al. 1989), studies have shown that, if eliminated upon administration of cytotoxic drugs like EDS,

the ALC (3β-HSD-positive) population is replenished from SLCs (3β-HSD-/LHR-/PDGFRA+ cells) in as little as 10 days in rats (Ge et al. 2006).

Aging In aging adults, declines in testosterone production occur (Harman et al. 2001). There is some debate if this decrease is due to a decline in ALC numbers (Neaves et al. 1985) or due to reduced steroidogenesis in individual cells (Chen et al. 1994). Zirkin and Chen (2000) demonstrated that reactive oxygen species from the Leydig cells themselves may be a source for ALC age-related testosterone decrease. Furthermore, degeneration of ALCs in aging adults by atrophy (i.e., changes in cytoplasmic volume), rather than dedifferentiation (Neaves et al. 1984), is supported by observations of classic aging pigments (lipofuscin granules), crystalline inclusions, decreased mitochondria, and diminished smooth endoplasmic reticulum (Mori et al. 1978, 1982; Paniagua et al. 1986).

5.4 Cellular Interaction with Leydig Cells

Leydig cells have complex interactions both locally and systemically which influence their survival, differentiation, and functionality (Fig. 5.5). Here we discuss their specific interactions with cells of the interstitium (including macrophages and vascular endothelial cells), as well as with cells of the seminiferous tubules (including germ cells, Sertoli cells, and peritubular myoid cells [PMCs]). However, the potential influence of FLCs on the generation of ALCs cannot be discounted, as ALC stem cells temporally overlap with FLC populations and, thus, the microenvironment during fetal and postnatal stages may influence ALC functionality later on (Kilcoyne et al. 2014); this interaction needs to be elucidated in further detail.

5.4.1 Cells of the Interstitium

Macrophages Testicular macrophages are intimately associated with Leydig cells. The interdigitation of slender Leydig cytoplasmic processes within macrophages forms deep channels (Christensen and Gillim 1969; Hutson 1992; Miller et al. 1983) and is thought to mediate proper ultrastructure in ALCs that is required for steroidogenesis (Cohen et al. 1997). This macrophage–Leydig interdigitation has been documented as early as 20–30 days postnatally in rats, just prior to pubertal testosterone secretion (Christensen and Gillim 1969; Hutson 1992; Miller et al. 1983). Their close proximity and cellular interdigitation has been thought to modulate the production of testosterone through lipophilic factors and/or cytokines (Hales 2002; Hutson 1998; Lukyanenko et al. 1998; Watson et al. 1994). Some cytokines (including interleukin-1, tumor necrosis factor-α, interleukin-6, transforming growth factor-β (TGFβ), interferon-γ, lipopolysaccharide, reactive oxygen species, and nitric oxide) are known to decrease steroidogenesis (reviewed

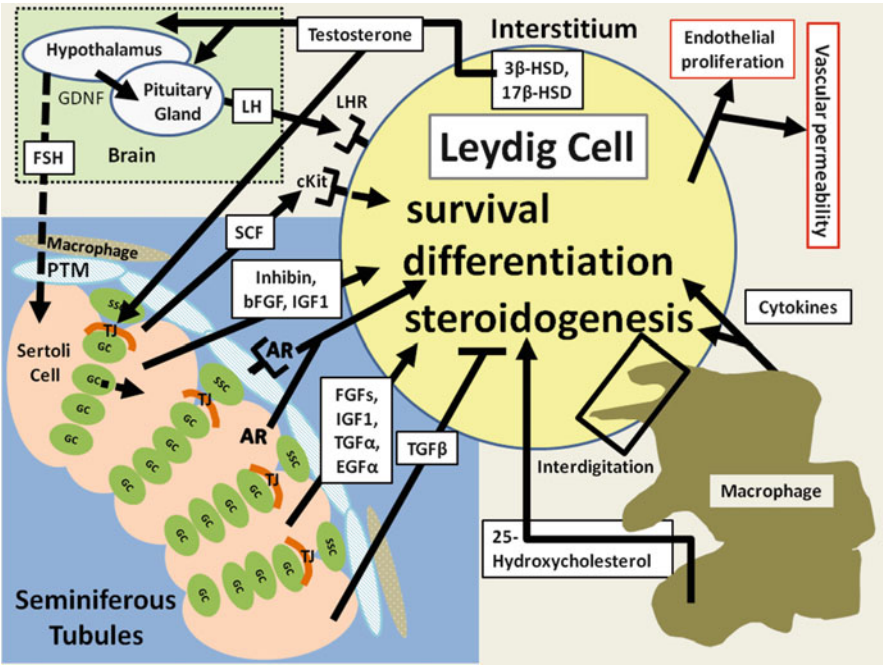


Fig. 5.5 Regulation of adult Leydig cell function. Cartoon depicting local and systemic factors that influence adult Leydig cell survival, differentiation, or steroidogenesis. *Dashed arrows* indicate long-distance or indirect interactions. Abbreviations are as follows: *AR* androgen receptor, *C-KIT* kit ligand/stem cell factor receptor, *EGFα* epidermal growth factor-α, *FGF-9* fibroblast growth factor-9, *bFGF* basic fibroblast growth factor, *GC* differentiated germ cell, *GDNF* glial cell line-derived neurotrophic factor, *IGF1* insulin-like growth factor, *LH* luteinizing hormone, *LHR* luteinizing hormone receptor, *PMC* peritubular myoid cell, *SCF* stem cell factor, *SSC* spermatogonial stem cell, *TGFα* transforming growth factor-α, *TGFβ* transforming growth factor-β, *TJ* tight junction

by Hales 2002), whereas unidentified lipophilic factors seem to increase testosterone production (Hutson 1998). Modulation of testosterone production has systemic effects and has been associated with changes in male mating behaviors, libido, and inter-male aggression (Hales 2002). It has been long known that macrophages directly secrete factors that positively influence steroidogenesis in Leydig cells (Yee and Hutson 1985); one of these candidate macrophage-derived factors has been identified as 25-hydroxycholesterol (Chen et al. 2002), an intermediate in the testosterone biosynthetic pathway (Nes et al. 2000). It is assumed that macrophage-secreted 25-hydroxycholesterol can be metabolized by Leydig cells and influences the capacity of Leydig cells to produce testosterone (Lukyanenko et al. 2001). Additionally, transient macrophage depletion (via a *Cx3cr1*-Cre-induced diphtheria toxin receptor ablation method) in the adult mouse testis does not disrupt Leydig cell numbers, but does decrease testosterone intratesticularly by half within 10 days

(DeFalco et al. 2015), showing that macrophage–Leydig cell interactions are dynamic and can rapidly affect steroidogenesis.

Testicular macrophages also play a role in adult Leydig cell development by providing growth and differentiation factors, as lack of macrophages leads to abnormally developed Leydig cells with altered functionality (Hales 2002); additionally, recovery of Leydig cells after EDS treatment is severely hindered in their absence (Gaytan et al. 1994b, c). Macrophages are important for ALC differentiation, as depletion of macrophages during critical windows in prepubertal rat development hindered Leydig cell differentiation (Gaytan et al. 1994a). Additionally, Leydig functionality may be influenced by macrophage activation status, as unstimulated macrophages inhibit testosterone in adult, but not immature, rats, whereas activated macrophages seemingly enhanced testosterone production in both adult and immature rats (Afane et al. 1998).

Vascular endothelial cells FLC progenitors are maintained via Notch signaling within a perivascular niche (DeFalco et al. 2013; Tang et al. 2008). Cells localized in the perivascular region are thought to be the stem cell source for ALCs that reconstitute the testis after Leydig cell depletion (Davidoff et al. 2004), although it is unknown if perivascular cells represent a common progenitor for other interstitial cells. Additionally, there is a knowledge gap in the field regarding the direct effects of vasculature on ALCs (e.g., differentiation, number, survival, and steroidogenesis). In the adult, Leydig cells are intimately localized to arterial and venous microvasculature, such as capillaries (Ergun et al. 1994). Conversely, Leydig cell activation via human chorionic gonadotropin (hCG) stimulation contributes to testicular vascular permeability (Collin and Bergh 1996; Setchell and Rommerts 1985) and endothelial cell proliferation (Collin and Bergh 1996). Given that ALCs localize near interstitial vasculature and that influential substances (e.g., luteinizing hormone [LH]) are delivered to ALCs from the systemic vasculature, it is likely that vasculature is critical for Leydig cell function. DeFalco et al. (2013) demonstrated that active Notch signaling, which is often associated with vasculature, was not detected in the interstitium of the adult testis; therefore, unlike FLCs, it is unlikely that vascular Notch signaling regulates ALC differentiation or function. However, alternative direct regulatory interactions have not been elucidated.

5.4.2 Cells of the Seminiferous Tubules

Germ cells Bergh (1982, 1983) previously hypothesized that germ cells influence Leydig steroidogenesis, due to differences in the size of Leydig cells associated with various stages of spermatogenesis. This correlation may be an indirect relationship, as other groups have demonstrated that germ cells do not play a role in Leydig cell steroidogenesis locally or systemically, utilizing both in vivo and cell culture germ-cell-depletion models (Chen et al. 2004; O'Shaughnessy et al. 2008; Sprando and Zirkin 1997; Wright et al. 1993). Germ cell influence on Leydig cells

is probably mediated through their modulation of Sertoli cells by controlling, among other factors, 17β-estradiol, transferrin, and inhibin (Carreau 1996), all of which influence testosterone production (Boujrad et al. 1992). Conversely, Leydig cells support germ cell development (Carreau et al. 2008; Hess 2003). This effect could be mediated through testosterone, as removal of testosterone blocks spermatogenesis at meiotic stages, and post-meiotic cells detach from Sertoli cells and undergo programmed cell death at the time of testosterone blockade.

Sertoli cells Adult Sertoli cells release factors important for Leydig cell survival (Rebourcet et al. 2014a, b), such as follicle-stimulating hormone (FSH), which is important for survival of both FLCs and ALCs (Benahmed et al. 1985); furthermore, loss of FSH receptors causes a decrease in Leydig cell numbers (Baker et al. 2003). Modulating AR expression in Sertoli cells has been shown to influence Leydig cell number and differentiation (Hazra et al. 2013). Additional paracrine activity of Sertoli cells (e.g., through inhibin subunits (Hsueh et al. 1987), basic fibroblast growth factor (bFGF) (Liu et al. 2014), and IGF1 (Hu et al. 2010) affects Leydig cell differentiation and influences steroidogenesis positively through fibroblast growth factor-9 (FGF-9) (Lin et al. 2010), basic fibroblast growth factor (bFGF) (Laslett et al. 1997; Liu et al. 2014; Sordoillet et al. 1992), insulin-like growth factor-1 (IGF1) (Gelber et al. 1992), epidermal growth factor-α (EGF-α), and transforming growth factor-α (TGF-α) (Millena et al. 2004), and negatively through transforming growth factor-β (TGF-β) (Lin et al. 1987). Testosterone influences Sertoli cell expression of Claudin-3, a tight junction protein comprising part of the blood–testis barrier (BTB), as well as affecting germ cell differentiation, thus having a major influence on fertility (Meng et al. 2005).

Peritubular myoid cells PMCs, a muscle cell type surrounding the tubules, influence adult Leydig cell development, ultrastructure, and function through the activity of AR. Mice lacking AR in PMCs had Leydig cells which were split into two groups: an unaffected, apparently "normal" group and an "abnormal" group, which displayed an arrested phenotype with lower levels of Insulin-like 3 (INSL3), LHR, and steroidogenic enzymes (Welsh et al. 2012), demonstrating the influence of PMCs on Leydig cells.

5.5 Interstitial Steroidogenesis

5.5.1 Normal Interstitial Steroidogenesis in the Male

Male interstitial steroidogenic cells Steroidogenic cells are located in the brain, placenta, adrenal glands, and the gonad. Regulation of steroidogenesis within the gonad is essential for sexual differentiation (Barsoum and Yao 2006), organ maintenance, and fertility (Meng et al. 2005). Testosterone, produced and released by ALCs and fetal/immature Sertoli cells (after production of androgenic

precursors by FLCs), functionally influences multiple aspects of male development: spermatogenesis (Walker 2011); the levels of gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH); male external genitalia differentiation (Tomiyama et al. 2003); and secondary sexual characteristics at puberty. Although steroidogenesis is commonly associated with local effects within the gonad, sex steroid hormones influence the maintenance of the whole body, including bone formation, metabolism, and erythropoiesis (Snyder et al. 2000).

Male interstitial steroidogenesis Steroidogenesis, the process of making steroid hormones, is performed within specialized cells, Leydig and Sertoli cells (male), and theca, granulosa, and luteal cells (female), within the gonad. Steroidogenic cells utilize enzymes to convert cholesterol into various hormones and hormone precursors. Steroidogenic acute regulatory protein (StAR), whose expression is restricted to steroidogenic organs, shuttles cholesterol across the mitochondrial membrane for processing, thereby regulating steroid production (Clark et al. 1994). This process requires LH stimulation of the Leydig cells to produce cyclic adenosine monophosphate (cAMP) to fuel movement of cholesterol across the membrane. Under the influence of gonadotropins, steroidogenesis is controlled through the production of steroidogenic enzymes, such as CYP11A1 (Hum and Miller 1993). Within the mitochondria, cholesterol is converted via CYP11A1 into cytosolic pregnenolone, which serves as a precursor to most sex hormones. Pregnenolone is further converted into progesterone via 3β-HSD (King and LaVoie 2009); the stepwise processing of the hormones from pregnenolone occurs within the smooth endoplasmic reticulum. The conversion of progesterone into androstenedione is performed by CYP17A1 (Gilep et al. 2011), followed by its final conversion into testosterone via 17β-HSD (Ge and Hardy 1998). Testosterone needs to be metabolically converted into dihydrotestosterone (DHT) via two isoforms of 5α-reductase (Ge and Hardy 1998).

Male-specific testosterone feedback loop Testosterone production is controlled systemically by the hypothalamic–pituitary–gonadal (HPG) axis. The hypothalamus produces gonadotropin-releasing hormone (GnRH), which induces the pituitary to produce the gonadotropins FSH and LH, which modulate Leydig cell function either directly or indirectly through other testicular cells, such as Sertoli cells (Krishnamurthy et al. 2001). LH signaling within Leydig cells stimulates the production of testosterone. Testosterone is then part of two feedback loops. The first is a systemic negative feedback loop, in which once a particular level of testosterone is reached, the hypothalamus will adjust the amount of GnRH produced, influencing LH, which in turn reduces testosterone output. The other feedback mechanism is local, in which Sertoli cells regulate the local pool of testosterone production in Leydig cells through releasing androgen-binding protein (ABP) which binds testosterone, thus keeping the concentration high locally; the local concentration of testosterone is 50–100-fold higher than systemic serum concentrations.

Stage-dependent male interstitial steroidogenesis Steroidogenesis in the testis occurs in a triphasic fashion in humans (Prince 2001), corresponding to the dynamics of the wavelike development and transitioning of distinct Leydig populations, thereby introducing fluctuations in testosterone production. Steroidogenesis is important for different reasons during various time periods in development. FLCs are required for the masculinization of the fetus, whereas the ALCs are essential for puberty and have important roles in spermatogenesis, libido, and overall male health. FLCs, the steroidogenic cells of the testis, appear within the mouse gonad by 12.5 *dpc*. By 13.5 *dpc*, FLCs are fully differentiated and express steroidogenic factor 1 (SF-1), which initiates steroidogenesis (Luo et al. 1994), and additionally express steroid biosynthesis enzymes, such as 3β-HSD, which are important for the production of androgens. FLC function is to convert cholesterol into androgen precursors, such as androstenedione. This is performed by acquiring exogenous cholesterol from sources outside the testis (Budefeld et al. 2009); the fact that cholesterol arrives to the testis via the vasculature is a possible reason why FLCs are localized to perivascular regions. Conversion of cholesterol to testosterone is gonadotropin-independent in rodents, as the HPG axis is not yet active during this time (O'Shaughnessy et al. 1998, 2006). FLCs do not contain a key steroidogenic enzyme, 17β-HSD, also called HSD17B3, which is required to convert androstenedione to testosterone; therefore, FLCs require the export of androstenedione to fetal Sertoli cells for the final processing of testosterone that is required for secondary sexual differentiation (O'Shaughnessy et al. 1998; Shima et al. 2013).

Postnatally, there is a transition period in which FLCs are still present, while the progenitors of ALCs are presumably present but have not started to differentiate. This period is followed by FLC degeneration, in which the production of testosterone reaches its lowest. After ALCs differentiate from ILCs, the 5α-reductase enzymes decrease, while 17β-HSD increases, switching the major output away from 5α-androstan-3α,17βdiol, a testosterone metabolite, toward testosterone within the testis. The conversion of cholesterol into testosterone in postnatal and adult stages occurs in a gonadotropin-dependent manner, as LH stimulation leads to increased testosterone production within ALCs. The importance of LH for ALC function not only lies in its stimulation of testosterone production; it also is required for the proliferation and differentiation of the ALC population (Baker and O'Shaughnessy 2001). ALCs have the capacity to produce more testosterone than FLCs, and, upon the onset of puberty, testosterone levels rise and are maintained when the organism reaches sexual maturity. This increase in testosterone is important, as testosterone deficit in hypogonadism patients is associated with multiple symptoms such as ambiguous genitalia, reduced masculinization (including decreased hair growth, decreased muscle mass, development of breasts), and infertility. In aged animals, testosterone production, both locally and systemically, starts to decrease (Harman et al. 2001), although the underlying cellular cause of this decrease is still under debate.

5.5.2 *Endocrine Disruptors of Male Interstitial Steroidogenesis*

Leydig cell steroidogenesis can be influenced by various environmental contaminants, including, but not limited to, pesticides, phthalates, industrial by-products, metals, and estrogenic compounds. The perturbation of Leydig cell function by these endocrine-disrupting compounds has relevance for human reproductive health, and uncovering their mechanism of action is a major area of research.

Pesticides One major class of pesticides, organochlorines, includes compounds such as *p*,*p*′-DDT (and its derivatives) and lindane (also known as gamma-hexachlorocyclohexane [γ-HCH]); these agents influence steroidogenesis (Enangue Njembele et al. 2014) by preventing cholesterol transport across the mitochondrial membrane via StAR (Walsh and Stocco 2000), conversion of the initial stages of testosterone synthesis from cholesterol to pregnenolone (Murono et al. 2006), or later stages by affecting 3β-, 17β-, and 11β-HSD enzymes (methoxychlor and 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane, also known as HPTE) (Guo et al. 2013; Hu et al. 2011). Some pesticides (e.g., procymidone, vinclozolin, linuron) modulate their effects on steroidogenesis via AR (Svechnikov et al. 2010). Pesticides can also influence StAR function by decreasing *StAR* expression levels transcriptionally (dimethoate and lindane) and through posttranscriptional modification (Glyphosate; commonly known as the commercial pesticide Roundup) (Walsh et al. 2000a, b; Walsh and Stocco 2000).

Phthalates Phthalate exposure can induce changes in FLC function (testosterone production), mitosis (number), and behavior (aggregation) or SLC proliferation and differentiation, all of which are hypothesized to lead to testicular dysgenesis syndrome (TDS) (Hu et al. 2009). Phthalates, commonly used in plastic manufacturing, influence Leydig cells in a stage-specific manner (Akingbemi et al. 2001; Rodriguez-Sosa et al. 2014) and in a species-specific manner (rat FLCs are sensitive, but mouse and human FLCs seem to be resistant) (Johnson et al. 2012). In rats, di(2-ethylhexyl)phthalate (DEHP) given postnatally (at 21–35 days old) caused significantly reduced 17β-HSD function and decreased testosterone output, whereas when given to adults (at 62–89 days old) there seemed to be no effect on androgen biosynthesis (Akingbemi et al. 2001).

Phthalate esters (such as DEHP and di-*n*-octyl phthalate [DOP]) perturb both the structure of Leydig cells and their testosterone output (Jones et al. 1993). Although some metabolites of phthalates (monophthalates) do not have any effect on Leydig cells (such as mono-*n*-butyl phthalate and mono-*n*-benzyl phthalate), others, such as mono-2-ethylhexyl phthalate (MEHP; the monoester of DEHP), have been demonstrated to influence mitochondrial function and stimulate basal steroidogenesis via StAR in postnatal mice (Savchuk et al. 2015). Others have shown that dibutyl phthalate (DBP) causes epigenetic modifications (via histone methylation) at the *StAR* promoter (Kilcoyne et al. 2014).

Industrial by-products Industrial by-products, such as dioxin, decrease size and number of Leydig cells (Johnson et al. 1994) and can influence cAMP levels (Lai et al. 2005) and cytochrome activity (Moore et al. 1991), thereby influencing steroidogenesis. Differential changes in steroidogenesis occur in a dose-dependent manner, as low doses of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (0.3 μg/kg) affect intratesticular testosterone, whereas higher concentrations (1 μg/kg) influence systemic feedback loops in rats (Adamsson et al. 2009). Another industrial by-product is benzophenones (BP). BP-1 is known to selectively inhibit 17β-HSD and therefore influences testosterone production (Nashev et al. 2010).

Metals Some metal cations (such as cadmium, cobalt, copper, mercury, nickel, and zinc) suppress hCG-induced or cAMP-induced testosterone production in Leydig cells (Laskey and Phelps 1991). This suppression may be through steroidogenic enzymes, such as 3β-HSD (arsenic, cadmium, chromium, lead, and mercury), 17β-HSD (arsenic and cadmium), CYP11A1 (lead), or via blocking StAR protein activity (cadmium, lead) (Payne and Hardy 2007).

Estrogenic compounds Testosterone can be metabolized into DHT (via 5-α-reductase) and estrogen (via aromatase). Since these two metabolites have opposing effects, disruption in the androgen:estrogen ratio can lead to infertility; therefore, a delicate balance is necessary for testicular function. Estrogen, acting through estrogen receptor alpha (ER-α), causes a reduction in *StAR*, *CYP17A1*, and *17β-HSD* steroidogenic gene expression, thereby influencing male-specific steroidogenesis (Strauss et al. 2009). Estrogen can be detrimental to Leydig cell development, as it blocks Leydig progenitor cell division when given between 5 and 30 days after EDS-induced Leydig cell depletion in rats; however, this blockade depends on the stage given, as when it is given at day 0–5 after EDS treatment it had no effect (Abney and Myers 1991). The xenoestrogen compound bisphenol A (BPA) has seemingly different effects on Leydig cells depending on the stage or species (N'Tumba-Byn et al. 2012). BPA disrupts immature Leydig steroidogenesis by affecting the conversion of testosterone into its metabolite 5α-androstan-3α,17βdiol, but does not affect basal androgen production (Savchuk et al. 2013) as it does in mature ALCs (Akingbemi et al. 2004). Differences were observed between rodent and human testis cultures: *INSL3* mRNA and testosterone production were reduced in humans, but not for rodents at low concentrations of 10(-8) M BPA (N'Tumba-Byn et al. 2012). Distinct signaling mechanisms between rodents and humans have been demonstrated, as nuclear estrogen receptor alpha, ERα, plays a role in BPA-reduced testosterone in rodents only, as fetal humans lack ERα (Gaskell et al. 2003) and seemingly signal through G-protein coupled receptor 30 and estrogen-related receptor gamma (Bouskine et al. 2009; N'Tumba-Byn et al. 2012).

5.6 Differing Properties of Male and Female Androgen-Producing Cells

The gonad initially arises as a bipotential organ during fetal development; thereafter, gonadal programming is required for sexual differentiation into the ovary and the testis. While some studies have suggested that the supporting cell types in the male and female gonad, the Sertoli and granulosa cells, respectively, arise from a common progenitor (Albrecht and Eicher 2001), not as much is known about the interstitial compartment of the gonad. The analogous interstitial androgen-producing cell type in the ovary corresponding to Leydig cells is the theca cell, which is localized around developing follicles. Similar to the origins of the adult testicular Leydig cells, theca cells are thought to arise from an adult stem cell progenitor, likely mesenchymal stromal precursor cells or steroidogenic cells with copious amounts of smooth endoplasmic reticulum. However, while Leydig cells commence steroidogenesis soon after initial testis formation, theca cells do not produce androgens until after birth upon LH stimulation when folliculogenesis begins (Mannan and O'Shaughnessy 1991; Fortune and Armstrong 1977).

While it is assumed that interstitial cells are recruited around the developing follicle due to paracrine signals from granulosa cells, the embryonic origin of theca cells is less clearly defined. Recent work has shown that, similar to Leydig cells, theca cells likely have multiple cellular origins: a subset of indigenous embryonic ovarian cells expressing *Wt1* (*Wilms tumor 1 homolog*) and migrating mesonephric mesenchymal cells expressing *Gli1* (encoding a member of the Hedgehog signaling pathway), which respond to Hedgehog signaling from granulosa cells (Liu et al. 2015). An early marker of fetal interstitial cells is the transcription factor MAFB; MAFB-expressing cells appear in the mouse gonad regardless of sexual differentiation around 12.5 *dpc* (DeFalco et al. 2011). Within the female, these cells reside near blood vessels, whereas their male counterparts not only localize to perivascular regions but also spread throughout the entire interstitial region (DeFalco et al. 2011). An additional putative marker for theca progenitors includes NR2F2, which is expressed in a unique subset of interstitial mesenchyme in the fetal ovary (Rastetter et al. 2014). Interestingly, NR2F2 is also expressed in Leydig cells and is required for Leydig cell differentiation during prepubertal stages (Qin et al. 2008), revealing a potential similarity between Leydig and theca cells.

Upon the development of the primary follicle, theca cells migrate and proliferate in the areas adjacent to the follicle (Eshkol and Lunenfeld 1972; Peters 1969). Granulosa-derived factors are thought to contribute to the androgen production and morphology of the theca interna cells (Kotsuji et al. 1990; Tajima et al. 2007). Although likely of similar cellular origin, different signaling pathways and genes likely regulate the differentiation of theca and Leydig cells; for example, *Gdf9* is required for theca cells alone, while Hedgehog signaling is required for proper function (i.e., steroid production) of both cell types (Clark et al. 2000; Dong et al. 1996; Liu et al. 2015). In the preantral follicle, Parrott and Skinner (1997) have shown that granulosa-derived SCF activates theca cell growth and

androstenedione production in cows, compared to a survival factor in Leydig cells. Although this is not an exhaustive list of factors, these examples demonstrate the heterogeneity of signaling influences between male and female androgen cells.

Both theca and Leydig cell androgen production are under the control of LH (Baird et al. 1981; Campbell et al. 1998; Palermo 2007; Ryan et al. 2008). Although LH/hCG and estrogen can stimulate theca cell steroidogenesis, additional factors, such as granulosa-derived activin, inhibin, BMPs, TGF-β, Gdf-9, and follistatin, can modulate hormone production (Tajima et al. 2007; Young and McNeilly 2010). Unlike ALCs, theca cells lack 17β-HSD, which is essential for conversion of androstenedione into testosterone, but do contain 3β-HSD (Sawetawan et al. 1994). Upon reaching the age of sexual maturity, Leydig cells reside in a post-mitotic phase with consistent numbers, whereas theca cells during ovulation become theca luteal cells, which produce massive amounts of progesterone until luteolysis (reviewed in Magoffin 2005). During and after luteolysis, theca luteal cells undergo apoptosis and become reabsorbed with the rest of the cells within the corpus albicans. An alternative fate of theca cells within atretic follicles is that they become hypertrophied and eventually lose their steroidogenic capacity (Himelstein-Braw et al. 1976; reviewed in Magoffin 2005).

5.7 Origin and Differentiation of Interstitial Steroidogenic Cells in Other Vertebrates

Vertebrates encompass a diverse set of animals, including amniotes (e.g., mammals, birds, and reptiles) and anamniotes (e.g., fish and amphibians), both of which possess reproductive organs but which have distinct testicular morphology. Amniotes exhibit a tubular structure interspersed with clumps of vasculature and interstitial cells, whereas the anamniotes have a cystic structure, either in the form of cysts or lobes. Originally thought to be lacking in anamniotes, Leydig cells have been observed in areas between the cystic regions (Norris and Carr 2013). Within the amniotes, seasonal and continuous/opportunistic breeders both have Leydig cells. Within the Leydig cells, the internal steroidogenic machinery, e.g., StAR, is evolutionarily conserved across species (i.e., piscine, avian, amphibian, and mammalian species) (Bauer et al. 2000).

Even among mammalian species, there are diverse gonadal phenotypes in terms of sex-specific steroidogenic cells. European mole (*Talpa occidentalis*) females have ovotestes that exhibit major fluctuations in steroid production and steroidogenic cell number in breeding versus nonbreeding seasons (Jimenez et al. 1993), likely to promote different behaviors appropriate for mating versus protection of offspring or territory. Similarly, spotted hyena females have high levels of androgen production (Browne et al. 2006), which drives female aggression that is evolutionarily advantageous due to the scavenger lifestyle of the hyena. However, this masculinization comes at great reproductive costs, as high levels of testosterone

result in a pseudoscrotum and elongated peniform clitoris which are ill-suited for the birthing process, so many females die during parturition (Browne et al. 2006; Dloniak et al. 2006; Lindeque and Skinner 1982).

Among certain species, Leydig cell function and breeding patterns vary seasonally. Rainfall, temperature, light, and nutrition all affect seasonal breeding patterns, likely through influencing androgen production. Changes in androgen biosynthesis by altered light cycle in hamsters are caused by changes in synthesis and secretion of testosterone, as well as direct regulation of circulating LH and Leydig cell responsiveness to LH signaling (Desjardins et al. 1971; Hardy et al. 1987). Opportunistic breeders, such as some rodents and humans, do not have seasonal changes in testicular weight once they reach sexual maturity (Suzuki and Racey 1978). Therefore, opportunistic breeders have relatively little change in their testis cellular populations until aging occurs. In contrast, seasonal breeders have months of recrudescence and intervening periods of regression, in which each period differs in testicular weight and spermatogenic activity. Within seasonal breeders, there are variations in how testis function is modulated: bats show changes in individual Leydig cell volume and Leydig cell granule number (Loh and Gemmell 1980), while stallions, birds, and amphibians exhibit changes in Leydig and Sertoli cell numbers instead of individual cell size (Johnson and Nguyen 1986; Johnson and Thompson 1986).

5.8 Conclusions and Future Areas of Research in the Field

While much research has been done regarding steroidogenic cells of the gonad, there are several outstanding questions in the field that are likely to be fruitful areas of future research. The first major topic deals with the origins of FLCs and ALCs. Indirect evidence points to several potential sources for the origin of FLCs and ALCs; however, which cell type(s) definitively gives rise to FLCs and ALCs still remains somewhat unresolved. Additionally, the physiological significance of the presence of two distinct populations of Leydig cells (fetal and adult) is unclear and requires further investigation. Another major unanswered question in the field is whether FLCs and ALCs arise from the same stem cell precursors or have distinct cellular origins. Finally, another topic of great interest to human health concerns the effects of endocrine-disrupting compounds on the differentiation and function of Leydig cells, as well as the subsequent impacts on male development. Answering these questions will provide a more comprehensive understanding of the molecular mechanisms of steroidogenic cell differentiation and its cellular origins in fetal and adult life. This knowledge will surely be of significance as it will help reveal the etiology of disorders of sexual development and will have implications for our understanding of fertility and other aspects of human health that are dependent on or influenced by gonadal steroid hormones.

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