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REVIEW

Fate determination of fetal Leydig cells

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Abstract **Leydig cell (LC) is one of the most important somatic cell types in testis, which localized in the interstitium between seminiferous tubules. The major function of Leydig cells is to produce steroid hormone, androgens. LC differentiation exhibits a biphasic pattern in rodent testes, which are divided into two different temporal mature populations, fetal Leydig cells (FLCs) and adult Leydig cells (ALCs). FLCs are transiently present in fetal testes and undergo involution or degeneration after birth. FLCs are completely devoid and replaced by ALCs in adult testes. Comparing to ALCs, FLCs display unique morphology, ultrastructure and functions. The origin of FLCs has been debated for many years, but it is still a mystery. Many factors have been reported regulating the specification, proliferation and differentiation of FLCs. FLCs degenerate in a few weeks postnatally, however, the underlying mechanism is still unknown. In this review, we will focus on the fate determination of FLCs, and summarize the resent progress on the morphology, ultrastructure, function, origin and involution of FLCs.**

Keywords fetal Leydig cells, adult Leydig cells, fate determination

Biphasic pattern of Leydig cell development

In mammals, the testis contains two distinct compartments, the seminiferous tubules and the interstitium. Sertoli cells (SCs) and germ cells (GCs) are two major cell types within the seminiferous tubule, the interstitium surrounds the seminiferous tubules and contains the steroidogenic Leydig cells, as well as the peritubular myoid cells (Habert et al., 2001). Leydig cells were named after the German anatomist Franz Leydig who first identified them in 1850. Leydig cells are somatic cells in the testicular interstitial space between seminiferous tubules that produce androgens. In 1904, fetal Leydig cells (FLCs) were first identified in pig embryos and a hypothesis was proposed simultaneously that two distinct LCs populations exist during testis development (Yao and Barsoum, 2007). Thereafter, the existence of two distinct populations of Leydig cells was found in other species, including rat, human, rabbit, mouse, pig, hamster, and ferret (Roosen-Runge and Anderson, 1959; Baillie, 1964; Niemi and Kormano, 1964; Lording and De Kretser, 1972; Gondos et al., 1977; Mendis-Handagama and Ariyaratne, 2001). FLCs appear in mice testis at embryonic day 12.5 (E12.5), and undergo involution or degeneration after birth. Their number is significantly decreased after birth and completely absent in mature testes (Fig. 1). Adult Leydig cells (ALCs) appear postnatally with distinct origin and morphology, which dominate the interstitial space after puberty (Faria et al., 2003; Haider, 2004; Haider et al., 2007).

Morphology and ultrastructure of FLCs

FLCs are arranged in clusters surrounded by a basal lamina (basement membrane) and a sheath of spindle-shaped fibroblasts at the outermost boundary at E16 in rat testes (Fig. 2). The major components of basal lamina are collagen type IV and laminin. The basal lamina is nearly continuous during fetal period and becomes discontinuous and patchy after birth. As steroidgenic cells, FLCs possess distinct ultrastructure with a large and round nucleus, a moderate-sized Golgi apparatus, abundance of smooth endoplasmic reticulum (SER), mitochondria and many large clustered lipid droplets (average diameter, 0.9 μm) (Fig. 3A). Cell membrane possesses numerous flat, finger-like, polyhedral, and inter-digitating protrusions. Many cell contacts are found between

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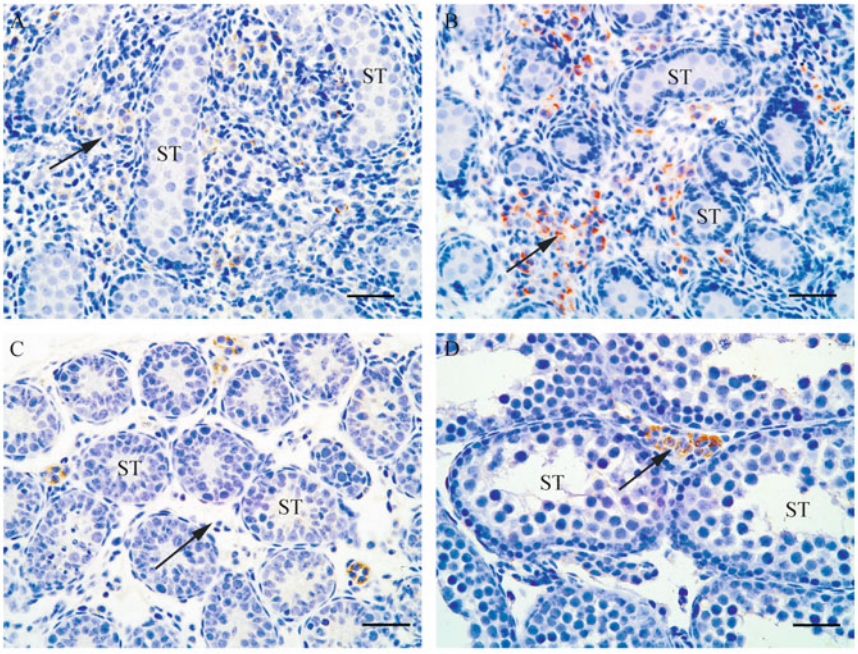

Figure 1 Leydig cell differentiation exhibits a biphasic pattern in mouse testes. Original magnification, × 400. Immunohistochemistry staining for Leydig cell marker, 3-beta–hydroxysteroid dehydrogenase (3β-HSD) A, E15.5 testis, FLCs are present in the testicular interstitium (arrow). B, postnatal day 1(D1) testis, more FLCs are noted in the testis (arrow). C, D7 testis, very few normal FLCs are found between the seminiferous tubules, most of them may undergo involution or degeneration postnatally (arrow). D, D21 testis, immature ALCs appear (arrow). ST, seminiferous tubule.

adjacent FLCs. Gap junctions and special desmosome-like cell contacts are observed between adjacent FLCs. Intercellular bridges with continuous cytoplasm are obvious between the FLCs within a cluster during the early postnatal period. ALCs contain large and round nuclei with one or two nucleoli, large amounts of SER, numerous tubulo-vesicular mitochondria, a well-differentiated Golgi apparatus, and relatively small lipid droplets (average diameter, 0.5 μm). Unlike FLCs, ALCs are not in cluster and basal membrane and broblasts are absent (Fig. 3B). Table 1 summarizes the main ultrastructural features of FLCs and ALCs (Haider, 2004; Haider et al., 2007).

The functions of FLCs

The main function of FLCs is to synthesize androgens and insulin-like factor 3 (INSL3 or relaxin-like factor). Androgens from FLCs are required for gonadogenesis, formation of ALCs precursors, regression of Mullerian duct, differentiation and morphogenesis of the male genital tract, and sexual "male imprinting" of the brain. Androgens and INSL3 also involve in the descent of testis (Yao and Barsoum, 2007). Whereas, androgens produced by ALCs are to initiate, maintain, and regulate the process of spermatogenesis (Haider, 2004; Yao and Barsoum, 2007).

Origin of FLCs

The origin of FLCs has been debated for decades, but it is still unclear. Four possible sources are proposed: 1) mesenchymal cells of gonadal ridge; 2) cells that migrate from the mesonephros; 3) coelomic epithelial cells which encapsulate the gonad; 4) cells that travel from neural crest and pericytes that invade developing testes with coelomic vessel.

Steroidogenic factor 1 (Sf1) is a transcription factor which is expressed in mesenchyme of gonadal ridge during testis development. Previous studies have demonstrated that Sf1 is essential for the specification of steroid-producing cells as well as for the expression of steroidogenic enzymes, Sf1-positive cells in gonadal ridge eventually differentiate into Sertoli cells, Leydig cells and other somatic cells. Sf1 deficient mice display defects in gonad development (Luo et al., 1994; Sadovsky et al., 1995; Hatano et al., 1996; Yao and Barsoum, 2007). This result further supports the idea that the Sf1-positive cell population in the gonadal primordium is one source of FLCs.

The gonadal primordium develops on the dorsal-medial part of the mesonephros along the anterior–posterior axis and establishes a close anatomical connection with the mesonephros (Yao and Barsoum, 2007). The results of gonad/mesonephros grafts and cocultures experiments indicate that

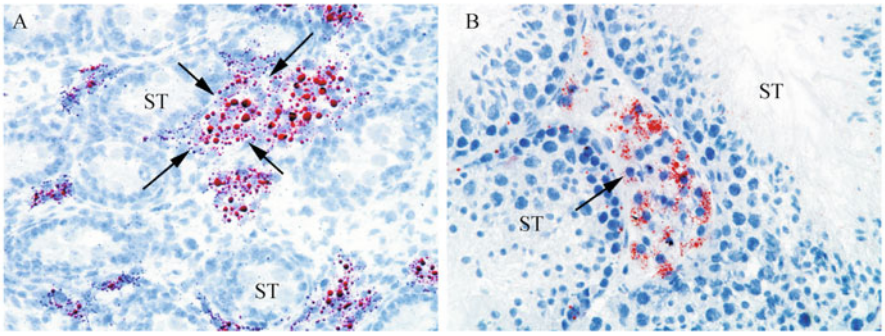

Figure 2 FLCs and ALCs in mouse testis. Original magnification, × 400. Oil Red O staining is performed on frozen section of D1 (A) and D56 (B) mouse testes. A, FLCs with characteristic large lipid droplets (red) are arranged exclusively in cluster (arrows). B, ALCs with small lipid droplets (red). ST, seminiferous tubule.

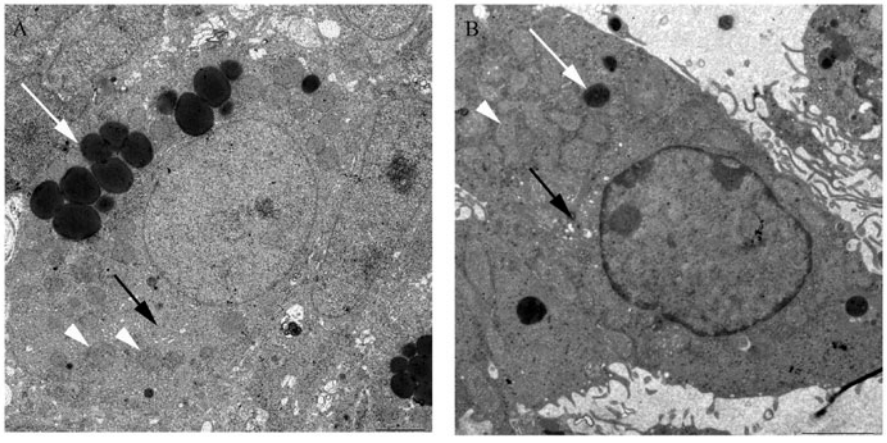

Figure 3 Ultrastructure of a mouse FLC and ALC. A, Original magnification, × 4000. FLC contains mitochondria with tubulo-vesicular cristae (white arrowheads), SER (black arrow), and large round clustered lipid droplets (diameter = 0.9 μm, white arrow). B, Original magnification, × 6000. A large amount of SER (black arrow), numerous mitochondria with tubulo-vesicular cristae(white arrowheads), and a few dispersed small lipid droplets (diameter = 0.5 μm, white arrow) are present in ALC.

Table 1 A comparison of ultrastructural features of fetal and ALCss

Features	In fetal LC	In adult LC	Common in both types
Nucleus	Round with smooth membranes	Elliptic or round with curly membranes	Thick euchromatin, numerous nuclear pores
SER	–	–	Present in abundance
Rough ER	Largely absent	Only a few	–
Mitochondria	–	–	Tubulo-vesicular cristae
Golgi apparatus	Small to moderate size	Large and well differentiated	–
Lipid droplets	Numerous (diameter = 0.9 μm)	A few (diameter = 0.5 μm)	–
Cell contacts	With other FLCs	With ALCs and fibroblasts	25 nm space contacts with adjacent LC
Microvilli	–	–	Accumulation in localized spaces
Surface	Numerous (finger-like protrusions)	A few, small protrusions	–
Basal lamina	Present (of various thickness)	Absent	–
Arranged	In clusters	Not exclusively in clusters	–

LC, Leydig cells; ALCs, adult Leydig cells; ER, endoplasmic reticulum; FLCs, fetal Leydig cells; SER, smooth endoplasmic reticulum (Haider, 2004).

Leydig stem cells ,first appear in the mesonephros, and then migrate into the presumptive interstitial tissue (Buehr et al., 1993; Martineau et al., 1997; Merchant-Larios and Moreno-Mendoza, 1998; Tilmann and Capel, 1999; Nishino et al., 2001). However, blocking the migration of the cells from mesonephros does not affect the differentiation of FLCs, suggesting that mesonephros is one source of FLCs, but not the only one (Yao and Capel, 2002).

Another possible source of Leydig cell precursors is the coelomic epithelium that covers the entire developing gonad ridge. Previous studies have revealed that coelomic epithelial cells rapidly proliferate in XY gonads between E11.5 and E12.5 and contribute to the interstitium of developing testis (Karl and Capel, 1998; Schmahl et al., 2000).

Several neural specific markers are expressed by Leydig cells, such as neural cell adhesion molecule (NCAM), neurofilament protein 200, and microtubular-associated protein. These phenomena lead to the hypothesis that Leydig stem cells come from neural crest (Angelova et al., 1991; Chiwakata et al., 1991; Mayerhofer et al., 1996). However, lineage tracing experiments using knockout mice models found no evidence of neural crest contribution to FLCs population (Brennan et al., 2003).

Testicular vasculature appears between E11.5 and E12.5 (Brennan and Capel, 2004), which is closely associated with the emergence of FLCs (Davidoff et al., 2009). Therefore, it is presumed that the pericyte, a source of stem cells of vascular wall, could also contribute to the FLCs (Brennan and Capel, 2004).

Taken together, the work has revealed that FLCs represents a cell type distinguished by a complex phenotype, covering epithelial, mesenchymal, myofibroblastic, endocrine and neuroendocrine characteristics. These evidences suggest that FLCs could derive from multiple sources (Yao and Barsoum, 2007; Davidoff et al., 2009).

Regulation of FLCs differentiation

The differentiation of FLCs occurs right after the formation of testicular cords. It is suggested that the appearance, differentiation, and maintenance of FLCs are regulated by the paracrine factors secreted by SCs and peritubular cells (Barsoum and Yao, 2010).

Desert hedgehog (Dhh) is one of the Hedgehog proteins that involves in a number of processes during embryonic development with Hedgehog signal pathway (Bitgood and McMahon, 1995). Dhh mRNA is first detected in Sertoli cells at E11.5 and its receptor Patched1 is prominently expressed in the interstitial cells of mouse testes at E12.5 and E13.5. In Dhh knockout mice, the number of Leydig cells is dramatically decreased at E13.5 and E14.5 of Dhh knockout testes. Further study demonstrates that the defects of Leydig cell differentiation in Dhh knockout XY gonads does not due to the failure of cell migration from the mesonephros, and the survival of fetal Leydig precursors in the interstitium of the XY gonad is also not affected, suggesting that Dhh/Patched1 signaling is essential for differentiation of FLCs (Yao et al., 2002). In addition, ectopically activation of the Hh pathway in Steroidogenic factor 1(SF1)-positive somatic cell of fetal ovaries results in the transformation of somatic ovarian cells into functional FLCs. These ectopic FLCs produced androgens and INLS3 that cause virilization of female embryos and ovarian descent. These results further demonstrate that Dhh/Patched1 signaling is necessary and sufficient for FLCs differentiation (Barsoum et al., 2009; Barsoum and Yao, 2010).

Platelet-derived growth factor A (PDGFα) which is required for embryonic and postnatal development is another factor which may involves in the differentiation of FLCs (Soriano, 1994; Boström et al., 1996; Betsholtz and Raines, 1997; Lindahl et al., 1998; Karlsson et al., 2000). PDGFα mRNA is strongly expressed in Sertoli cells of E12.5 testes. Its receptor, PDGFR-α, is expressed in mesenchyme of mesonephrosand coelomic epithelium at E11.5. At E12.5, high expression of PDGFR-α is noted in interstitial cells of XY gonad, particularly near the coelomic epithelium. PDGFR-α deletion in male mice results in severe defects in Sertoli cell proliferation, mesonephric cell migration, and FLCs differentiation. These results support the presumption that PDGFα/PDGFR-α signaling is necessary for proliferation, differentiation and migration of FLCs precursors (Brennan et al., 2003).

ARX is a transcription factor encoded by the X-linked aristaless-related homeobox gene. It is not expresssed in FLCs whereas very strong signal is detected in fibroblast-like cells of testis interstitium. ARX knockout mice display severe defect of FLCs population, this result suggests that ARX is essential for establishment of stem FLCs (Kitamura et al., 2002).

Pod1 (capsulin/epicardin/Tcf21) is a basic helix–loop–helix transcription factor which is expressed primarily to the coelomic epithelium of gonad and the boundary region between the gonad and mesonephros at E11.5. At E18.5, the expression of Pod1 is noted in peritubular myoid cells surrounding the testis cords, presumed Leydig cells in the interstitial region, and pericytes surrounding capillaries. In Pod1 deficient testes, the number of cholesterol side-chain cleavage enzyme (Scc) expressing cells is increased markedly and the expression of Sf1 is elevated in Pod1 deficient testes, implying that Pod1 may involve in FLCs differentiation by repressing Sf1 transcription in FLCs progenitors (Cui et al., 2004).

Notch, a transmembrane receptor that mediates local communication between cells, is involved in cell fate determination, particularly in stem cell maintenance and differentiation in many animal systems (Lai, 2004). Four Notch receptors (Notch1–Notch4) and five structurally similar Notch ligands, delta-like 1 (also called Delta1), delta-like 3, delta-like 4, jagged 1 and jagged 2 are found in

mammals (Schroeter et al., 1998; De Strooper et al., 1999; Huppert et al., 2000). Hes1 and Hes5, the hairy/enhancer of split genes, are the most well-defined targeting genes of Notch signal pathway (Kageyama et al., 2007). Notch2 is detected at low levels in the coelomic epithelium and in deeper layers of the XY gonad at E11.5. Notch2 expression is localized to pre-Sertoli and Sertoli cells during the testicular cords formation betwen E12 and E12.5. At E13.5, Notch2 expression decline in Sertoli cells, and shifted to the interstitium. Notch3 is expressed in the interstitium of the XY gonad between E12.5 and E13.5. Hes5 is detected in Sertoli cells inside testis cords, whereas Hes1 expression is restricted to interstitium at E12.5, suggesting that Notch signaling pathway is active in sertoli cells and interstitial cells of testes. Blocking Notch signaling by inhibiting γ-secretase activity or deleting the downstream target gene Hes1 results in an increase number of Leydig cells in the testis. In contrast, constitutively active Notch signaling in gonadal somatic progenitor cells causes loss of FLCs, however, the number of undifferentiated mesenchymal cells is increaseed. These results indicate that active Notch signaling may repress the differentiation of FLCs (Tang et al., 2008).

Anti-Mullerian hormone (AMH), also called Mullerian inhibiting substance (MIS) or factor (MIF) belonging to the transforming growth factor-β (TGFβ) family and is responsible for the regression of Mullerian ducts in male fetuses, which is produced by Sertoli cells from the time when the testicular seminiferous cords differentiate until pubertal maturation and also by postnatal granulose cells (Lee and Donahoe, 1993). Whereas, its receptor is present in progenitor, immature and mature ALCs and FLCs (Baarends et al., 1994; di Clemente et al., 1994; Baarends et al., 1995; Teixeira et al., 1996; Racine et al., 1998; Lee et al., 1999). AMH deficient male mice develop focal Leydig cell hyperplasia (4 out of 15) over 10 weeks of age, and one mouse reveals a Leydig cell tumor. It is reported that most of the AMH transgenic male mice develop normally and are fertile. In two lines with high level of AMH show external genitalia feminization, Wolffian duct development impairment, and undescended testes (Behringer et al., 1990). In addition, the number of immature and mature ALCs is decreased whereas the number of mesenchymal cells, which include ALCs precursors, is increased in AMH transgenic male mice (Racine et al., 1998). These results suggest that AMH may negatively regulate LCs proliferation (Behringer et al., 1994; Mishina et al., 1996).

Perspective

This review summarizes the recent progress on the morphology, function and origin of FLCs. More attention is paid on illuminating the signalings that regulate the specification, proliferation and differentiation of FLCs. Even though great progress has been made in this field recently, many uncovered mysteries are still remaining, i.e. the factors which regulating the involution of FLCs are still unknown, and the physiologic significance of existing two population of LCs also needs further investigations. The origin of FLCs has been extensively studied, however, none of the hypothsis has been verified. All of these questions need to be addresses in the future research.

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