Androgen profiles during pubertal Leydig cell development in mice

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Abstract

Postnatal Leydig cell (LC) development in mice has been assumed empirically to resemble that of rats, which have characteristic hormonal profiles at well-defined maturational stages. To characterize the changes in LC function and gene expression in mice, we examined reproductive hormone expression from birth to 180 days, and quantified *in vivo* and *in vitro* production of androgens during sexual maturation. Although the overall plasma androgen and LH profiles from birth through puberty were comparable to that of rats, the timing of developmental changes in androgen production and steroidogenic capacity of isolated LCs differed. In mice, onset of androgen biosynthetic capacity, distinguished by an acute rise in androstenedione and testosterone production and an increased expression of the steroidogenic enzymes, cytochrome P450 cholesterol side-chain cleavage enzyme and 17 α -hydroxylase, occurred at day 24 (d24) rather than at d21 as reported in rats. Moreover, in contrast to persistently high testosterone production by pubertal and adult rat LCs, testosterone production was maximal at d45 in mice, and then declined in mature LCs. The murine LCs also respond more robustly to LH stimulation, with a greater increment in LH-stimulated testosterone production. Collectively, these data suggest that the mouse LC lineage has a delayed onset, and that it has an accelerated pace of maturation compared with the rat LC lineage. Across comparable maturational stages, LCs exhibit species-specific developmental changes in enzyme expression and capacity for androgen production. Our results demonstrate distinct differences in LC differentiation between mice and rats, and provide informative data for assessing reproductive phenotypes of recombinant mouse models.

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Introduction

During rodent testis development, Leydig cells (LCs) arise as two discrete but overlapping populations, fetal and adult type. During embryogenesis, androgens produced by the fetal LCs are essential for masculinization. At birth, the fetal LC population declines dramatically in number by undergoing apoptotic cell death or dedifferentiation (Gondos *et al.* 1977, Kuopio *et al.* 1989), although residual fetal LCs have been identified in the pubertal testis (Kerr & Knell 1988). Adult-type LCs, which arise from mesenchymal precursor cells, start to differentiate at postnatal day 10 (d10)–d14 in rats and at d7–d10 in mice (Mendis-Handagama *et al.* 1987, Hardy *et al.* 1989, Vergouwen *et al.* 1991, Nef *et al.* 2000), and then gradually mature to adult LCs. Terminal differentiation of postnatal LCs is needed at puberty and in adults for the production of testosterone, which is essential for secondary sexual maturation, male mating behavior, and fertility.

In rats, the development of differentiating adult-type LCs has traditionally been divided into three distinct

maturational stages: progenitor, immature, and adult (Shan *et al.* 1993, Benton *et al.* 1995). PLCs are present from d14 to d28, and then differentiate into ILCs from d28 to d35 and fully mature to ALCs by d56 (Shan *et al.* 1993, Benton *et al.* 1995). Each sequential stage exhibits characteristic cellular structural features and androgen profiles. Rat PLCs proliferate actively, and start to express androgen biosynthetic enzymes, cytochrome P450 cholesterol side-chain cleavage enzyme (P450_{scc}), and 17 α -hydroxylase (P450_{c17 α}). Owing to the low expression of steroidogenic enzymes that are present abundantly in more mature LCs, i.e. type III 17 β -hydroxysteroid dehydrogenase (17 β Hsd III) and type VI 3 β -hydroxysteroid dehydrogenase (3 β Hsd VI), the predominant androgens produced at this stage are androstenedione and androsterone (Shan *et al.* 1993, Benton *et al.* 1995, Ge & Hardy 1998). By d28, PLCs differentiate into ILCs in rats, which express high levels of both androgen biosynthetic and metabolizing enzymes, making androstenediol the most abundant androgen. By d56, the ILCs terminally differentiate into ALCs that still express type III 17 β Hsd abundantly (Ge & Hardy 1998),

while the expression of androgen metabolizing enzymes is downregulated, thereby leading to the highest net production of testosterone (Shan *et al.* 1993, Benton *et al.* 1995, Viger & Robaire 1995). These stage-specific androgen profiles reflect the opposing actions of androgen biosynthetic and metabolizing enzymes (Shan *et al.* 1993, Ge & Hardy 1998, Payne & Hales 2004), and demarcate maturational transitions in LC development and differentiation (Shan *et al.* 1993, Ge *et al.* 1996, Wu *et al.* 2007).

Although the developmental progression of rat LC differentiation has been well characterized, these transitions have not been fully defined using primary LCs in mice. With the increasing use of genetically engineered mouse models, an accurate delineation of the normal developmental maturation of mouse LCs will facilitate the ability to recognize perturbations in LC development and function. Therefore, in this study, we have characterized and defined the transitional changes and hormonal profiles at sequential stages of postnatal LC development in mice.

Results

Plasma hormone concentrations

Plasma LH concentrations were low after birth until d14, and then started to increase at d21–d28 and became significantly higher at d35 (Fig. 1A). Mean LH increased further to a peak value at d45, and then remained

elevated from this age onwards. After birth, plasma testosterone concentrations declined to a nadir at d21, and then started to increase at d24 and reached a significantly higher value at d35 (Fig. 1A). Plasma testosterone subsequently rose to peak concentrations at d45, and remained elevated thereafter.

Intratesticular androgen concentrations

Intratesticular concentrations of androstenedione, androstanediol, and testosterone were low from birth to d14 (Fig. 1B–D). At d24, intratesticular androstenedione first became significantly higher than that observed at younger ages and continued to rise through d45, and then remained elevated (Fig. 1B). Intratesticular androstanediol concentrations increased above the prepubertal range at d21, peaked at d35, and then declined to lower values from d45 to d180 (Fig. 1C). Intratesticular testosterone concentrations increased slightly at d7, and then progressively increased to d45 and remained elevated thereafter (Fig. 1D).

In vitro basal androgen production

Both basal and LH-stimulated *in vitro* production of androgens by cultured primary LCs were examined (Fig. 2). Basal androstenedione synthesis increased 10-fold from d21 to d24 (18.13 ± 0.83 vs 199.49 ± 2.99 pmol/million cells, $P < 0.001$) and progressively increased until d45, and then declined significantly from

Figure 1 Plasma LH and testosterone concentrations. For mice younger than d14, plasma was pooled from three mice, and testicular extracts were pooled from 12 testes. At all other ages, individual plasma samples and testicular extracts were assayed. Each data point represents the mean ($\pm$ S.E.M.) of three sets of pooled samples or 3–9 individual samples run in duplicate. Different superscripts denote significant differences ($P < 0.05$). ND, not done. (A) Plasma LH starts to rise at d21 and then increases progressively to d45, while plasma testosterone increases from d24 to d45, and is elevated through d180. *Plasma LH on d35 is significantly greater than that on d1–d14. **Plasma LH from d45 onwards is significantly greater than that observed at all other ages. (a) Plasma T on d7 is significantly greater than that on d21. (b) Plasma T on d35 is significantly greater than that on d21. (c) Plasma T from d45 onwards is significantly greater than that observed at all other ages. (B) Intratesticular androstenedione initially increases above prepubertal values at d24 ($P < 0.05$), and then rapidly rises until d45 and remains high through d180. (C) Intratesticular androstanediol increases at d21, and then remains unchanged except for a slight peak at d35. (D) Intratesticular testosterone progressively increases from d24 through d45, and then remains elevated.

Figure 2 Basal (A–C) and LH-stimulated (D–F) *in vitro* androgen production by primary Leydig cells. Primary LCs were cultured for 3 h with 0.07 nM (1 ng/ml) and 7 nM (100 ng/ml) LH. Conditioned media from primary LCs were assayed for androstenedione, androstenediol, and testosterone. Each data point (mean $\pm$ S.E.M.) represents triplicate determinations of Leydig cells from 6 to 20 pooled testes. Different superscripts denote significant differences ($P < 0.05$). ND, not done. UN, undetectable values (below assay detection limits). Basal androgen production: (A) androstenedione in conditioned media increases above the prepubertal range at d24, rises steadily to a peak at d45, and then declines precipitously at d60. (B) Androstenediol in conditioned media dramatically increases from d24 to 35, and then declines to lower values by d45. (C) Testosterone in conditioned media first rises at d24, and then increases to peak at d45 with a rapid decline thereafter. LH-stimulated androgen production: (D) androstenedione increases at d24, reaches peak values at d35 and d45, and declines by d60. (E) Androstenediol is highest on d28, and then declines with another peak at d75. (F) Testosterone is first measurable at d24, and then progressively increases to a peak value at d75 before declining markedly at d90.

d60 onwards (Fig. 2A). The *in vitro* production of androstenediol was detected at d7 and was maximal at d35, and then declined to much lower levels from d45 to d90 (Fig. 2B). *In vitro* basal testosterone production was first measurable at d24, peaked at d45, and then decreased precipitously at d60 and continued to progressively decline thereafter (Fig. 2C).

In terms of basal androgen production, androstenedione was the predominant androgen produced at d24 (199.49 ± 2.99 pmol/million cells per 3 h compared with 40.07 ± 4.17 pmol/million cells per 3 h for androstenediol and 86.13 ± 7.75 pmol/million cells per 3 h for testosterone). In contrast, testosterone was the most abundant androgen produced at d45 (1117 ± 86.15 pmol/million cells per 3 h compared with 281.59 ± 26.66 pmol/million cells per 3 h for androstenedione and 33.46 ± 2.59 pmol/million cells per 3 h for androstenediol). Thus, the immature androgen, androstenedione, comprised 61% of the total androgens produced at d24, whereas a switch in the androgen production was noted by d45, when testosterone comprised 78% of the total androgen production.

LH-stimulated *in vitro* androgen production

The LH-stimulated androstenedione production by cultured primary LCs increased 11-fold from d21 to d24 (from 28.45 ± 0.48 to 320.11 ± 4.28 pmol/million cells per 3 h, $P < 0.001$) and continued to progressively increase until d45, and then declined from d60 onwards (Fig. 2D). The LH-stimulated production of androstenediol was detectable at d7, reached a maximum at d28, and then declined significantly by d45 with an isolated spike at d75 (Fig. 2E). LH-stimulated testosterone production was not detectable until d24, and then gradually increased to peak at d75, with a dramatic decrease at d90 (Fig. 2F).

With LH stimulation, androstenedione was the predominant biosynthetic androgen produced at d24 (320.11 ± 4.28 pmol/million cells per 3 h compared with 78.45 ± 8.09 pmol/million cells per 3 h for androstenediol and 190.3 ± 30.25 pmol/million cells per 3 h for testosterone). In contrast, testosterone was the most abundant androgen produced at d45 (2540 ± 38.27 pmol/million cells per 3 h compared with 967.32 ± 30.97 pmol/million cells per 3 h for

androstenedione and 23.87 ± 1.71 pmol/million cells per 3 h for androstenediol). Consequently, at d24, androstenedione production comprised 54.4% of the total androgen production, whereas at d45, there was a shift to the predominant synthesis of testosterone, which comprised 71.9% of the total androgen production. The ability of LH to stimulate LC androgen production appeared to be acquired between d21 and d24. At d21, testosterone production did not rise in response to LH stimulation, whereas by d24, testosterone production increased 2.2-fold (from 86.13 ± 7.75 to 190.3 ± 30.25 pmol/million cells per 3 h).

Steroidogenic enzyme expression

The mRNA expression of androgen biosynthetic and metabolic enzymes was examined by northern blot analysis in total testicular RNA and by quantitative real-time PCR (QPCR) in RNAs extracted from purified mouse LCs. By northern blot analysis, P450scc and P450c17 α are detectable in total testis by d7 at low levels (most likely representing fetal LCs; Fig. 3A and B). Both RNAs are abundantly expressed from d24 to d60, and then decline in abundance thereafter. Using ImageJ software (<http://rsb.info.nih.gov/ij/>), the mRNA bands for each time point were expressed as the ratio of P450scc/S16 and P450c17 α /S16 (Fig. 3B).

By QPCR, the metabolic enzymes, 3 α Hsd and 5 α -reductase, were expressed most abundantly in mouse LCs at d21, and then decreased with subsequent maturation (Table 1). In contrast, the steroidogenic enzymes, 17 β Hsd III, P450c17 α , and StAR, were expressed at lower levels at d21 and d24, and then increased significantly at d35–d90 (Table 1). The mRNA expression of P450scc, by both northern blot analysis and QPCR, increased from d24 to d60, and then declined. The mRNA expression of 3 β Hsd and 3 β Hsd VI did not vary from d24 to d60 (data not shown). The mRNA expression of the LH receptor significantly rose from d14 (data not shown) to d35, and then remained abundantly expressed thereafter with minor fluctuations (Table 1).

To confirm that developmental changes in the mRNA expression of the steroidogenic enzymes at the key peripubertal transition from d21 to d24 were accompanied by parallel changes in protein expression, immunohistochemistry was performed on testicular sections. These studies showed an obvious increase in the number of LCs with positive staining for both P450scc and P450c17 α from d21 to d24 (Fig. 4A and B).

Discussion

The data demonstrate the developmental changes in murine LC differentiation and androgen production from birth through maturity. In comparison to published data obtained from rats, our data obtained from mice reveal

Figure 3 The mRNA expression of androgen biosynthetic and metabolic enzymes. (A) Total RNA was extracted from mouse testes collected from d7 to d90 for northern blot analysis. Ribosomal protein S16 was used as an internal control. P450scc and P450c17 α mRNAs are first detectable at d24, and are then abundantly expressed until d60. (B) The above northern blot was quantified using ImageJ software, and each time point was expressed as the ratio of P450scc/S16 and P450c17 α /S16 (mean $\pm$ S.E.M.).

species differences in developmental androgen profiles and in the timing and pace of the maturational transition from an undifferentiated prepubertal LC to a sexually mature adult LC. This study demonstrates the differences between rats and mice in the response of LCs to LH stimulation and in the maturational profiles of steroidogenic and metabolic enzymes.

The general pattern of intratesticular androgen concentrations and steroidogenic enzyme mRNA expression across the pubertal transition is similar to that previously reported (Baker *et al.* 1997, 2003, O'Shaughnessy *et al.* 2002). In contrast to these studies, we examined steroidogenic enzyme expression in RNA isolated from purified LCs rather than that in RNA isolated from total testis or testicular webs, which includes a mixed population of cells (Baker *et al.*

Table 1 The mRNA gene expression in primary Leydig cells from d21 to d90.

Ages	Lhr	StAR	P450scc	P450c17 α	17 β Hsd III	3 α Hsd	5 α -reductase
21	0.67 $\pm$ 0.03 ^{†,‡}	0.60 $\pm$ 0.25*	4.12 $\pm$ 0.27* [†]	3.78 $\pm$ 0.19*	0.01 $\pm$ 0.001*	0.04 $\pm$ 0.002*	0.36 $\pm$ 0.018*
24	0.57 $\pm$ 0.03 ^{‡,§}	0.45 $\pm$ 0.14*	2.57 $\pm$ 0.16*	3.07 $\pm$ 0.59*	0.01 $\pm$ 0.001*	0.03 $\pm$ 0.007 [†]	0.34 $\pm$ 0.017*
35	0.96 $\pm$ 0.06*	1.26 $\pm$ 0.45 [†]	7.55 $\pm$ 0.14 ^{‡,§}	8.15 $\pm$ 0.58 [†]	0.12 $\pm$ 0.011 [†]	0.03 $\pm$ 0.001 [†]	0.14 $\pm$ 0.003 [†]
45	0.80 $\pm$ 0.04* [†]	1.47 $\pm$ 0.59 [†]	9.12 $\pm$ 0.60 [§]	8.14 $\pm$ 0.64 [†]	0.15 $\pm$ 0.005 ^{†,‡}	0.01 $\pm$ 0.001 [‡]	0.18 $\pm$ 0.005 [†]
60	0.68 $\pm$ 0.06 ^{†,‡}	1.52 $\pm$ 0.48 [†]	11.47 $\pm$ 0.77 [§]	10.25 $\pm$ 0.30 [†]	0.26 $\pm$ 0.026 [§]	0.02 $\pm$ 0.001 [§]	0.04 $\pm$ 0.009 [‡]
90	0.38 $\pm$ 0.06 [§]	1.29 $\pm$ 0.45 [†]	5.91 $\pm$ 0.13* ^{‡,‡}	8.64 $\pm$ 0.21 [†]	0.20 $\pm$ 0.019 ^{‡,§}	0.01 $\pm$ 0.001 ^{‡,§}	0.02 $\pm$ 0.005 [‡]

Each data point (mean $\pm$ S.E.M.) represents the mean mRNA expression normalized to actin from at least three separate Leydig cell isolations and RNA extractions. Different superscripts denote significant differences ($P < 0.05$).

1997, 2003, O'Shaughnessy *et al.* 2002). We also examined steroid production and LH responsiveness in highly purified primary LC preparations rather than that in non-purified interstitial or testicular fractions (Sheffield & O'Shaughnessy 1988, O'Shaughnessy *et al.* 2002), which enabled us to quantify androgen production per given LC number and permitted more precise delineation of maturational changes in steroidogenesis. Studies using whole testicular extracts to examine enzymatic activity can reflect steroidogenic enzyme activity in Sertoli as well as LCs (Welsh & Wiebe 1978, Sheffield & O'Shaughnessy 1988). Moreover, the use of RNA from total testes or interstitial webs rather than that from LCs results in variability in the relative fraction of LC-specific RNA as the proportion of each cell type changes during pubertal maturation (Vergouwen *et al.* 1993). While overall changes in steroidogenic enzyme expression and androgen production exhibited similar profiles among the studies, discrete differences in amplitude or timing may reflect these methodological differences or perhaps strain differences.

The gradual rise in intratesticular concentrations of androstenedione and testosterone and the increasing responsiveness of isolated LCs to LH stimulation at the onset of puberty are comparable to previous reports in mice (O'Shaughnessy *et al.* 2000) and rats (Ge & Hardy 1998, Mendis-Handagama *et al.* 1998). The predominant production of androstenedione at pubertal onset is due to the increased capacity of postnatal LCs to synthesize androgens as they differentiate from mesenchymal precursors and acquire expression of the steroidogenic enzymes. At this stage, the immature LCs abundantly express 5 α -reductase and 3 α Hsd, but exhibit a much lower expression of 17 β Hsd III, a pattern generally consistent with that reported previously (Shan *et al.* 1993, Baker *et al.* 1997, Ge & Hardy 1998, O'Shaughnessy *et al.* 2002, Dong *et al.* 2007). Consequently, androstenedione and androstenediol rather than testosterone are produced preferentially by the immature peripubertal LCs. Androstenedione production by LCs continues to increase after d45 in rats, although constituting a smaller fraction of the total androgens produced (Ge & Hardy 1998, Ariyaratne & Chamindrani Mendis-Handagama 2000), while in mice, androstenedione synthesis by LCs declines dramatically

(Wu *et al.* 2005). Despite the decreased androstenedione production by isolated LCs, our data also confirm that intratesticular and plasma concentrations of androstenedione remain elevated in adult mice (O'Shaughnessy *et al.* 2000, Wu *et al.* 2005). This ability to maintain high concentrations of androstenedione despite a decreased *in vitro* capacity per LC for androstenedione synthesis could be explained by the presence of a higher percentage of immature LCs in the mouse testis than in the rat testis at these ages. In rats at comparable ages, there is minimal proliferation or differentiation of LCs as they have been shown to be terminally differentiated (Benton *et al.* 1995). Our preliminary data show a similar mitotic index in adult and prepubertal dividing mouse LCs by flow cytometry (X F Wu & M M Lee, unpublished data). We hypothesize that continued recruitment of adult-type LCs from mesenchymal precursors is ongoing in the mouse testis, and that these newly maturing LCs make a substantial contribution to the type of androgens produced at these ages.

During pubertal development, a number of factors influence LC differentiation and steroidogenesis (Ge *et al.* 1996, Wu *et al.* 2007). Androstenediol reflects metabolic enzyme activity and is the major androgen produced by immature rat LCs (Benton *et al.* 1995, Ge & Hardy 1998). Intratesticular concentrations of androstenediol peak in the pubertal mouse testis, and then decline with sexual maturation. In mice, *in vitro* production of LH-stimulated androstenediol was maximal between d28 and d35, and then decreased at older ages (Sheffield & O'Shaughnessy 1988, O'Shaughnessy *et al.* 2000) similar to that observed in rats (Benton *et al.* 1995, Killian *et al.* 2003). These developmental changes during testicular maturation in mice parallel the report in rats of increased activity of the metabolic enzymes during puberty followed by a subsequent decline in mature LCs (Shan *et al.* 1993). The similar patterns of androgen production and developmental changes in 3 α HSD and 5 α -reductase expression we observed in mice suggest that corresponding changes are at play during murine LC maturation. Therefore, the significant decline in androstenediol production and expression of 3 α Hsd from d35 to d45 may denote a maturational transition in the differentiation of the mouse LC towards a terminally differentiated mature state.

Figure 4 Immunohistochemistry of P450scc and P450c17 α expression in mouse testis. Staining for both P450scc (A) and P450c17 α (B) in testicular sections increases markedly from d21 to d24. Five-micrometre sections from three different mouse testes were examined.

Net testosterone production is dependent on both the balance of androgen metabolic and biosynthetic enzyme activity and the number of steroidogenically active LCs in the testes. In our mouse study, plasma and intratesticular testosterone concentrations, a steady-state measurement reflecting net actions of androgen biosynthetic and metabolizing enzymes, were high from d45 onwards, a pattern similar to that reported in mice (O'Shaughnessy *et al.* 2000, 2002, Wu *et al.* 2005) and rats (Ge & Hardy 1998, Ariyaratne & Chamindrani

Mendis-Handagama 2000). As rat LCs mature, a decrease in the expression of the enzymes that metabolize testosterone leads to increased net production of testosterone by ALCs (Ge & Hardy 1998, Dong *et al.* 2007). Conversely, despite increasing concentrations of intratesticular testosterone, we found a decline in both basal and LH-stimulated testosterone production by isolated LCs at d60 and d90 respectively. We speculate that this discrepancy, i.e. increased testicular testosterone concentrations in the adults versus

decreased *in vitro* testosterone production, may reflect an increase in LC numbers that compensates for the decreased testosterone biosynthetic capacity per cell.

Our study has characterized androgen secretory profiles during murine reproductive development, and defined the biosynthetic capacity and LH responsiveness of maturing LCs. These data enabled us to clarify the maturational transitions when LCs become initially recognizable as progenitor cells, differentiate into immature LCs, and then terminally differentiate into adult LCs in mice. The initial evidence of puberty is a modest increment in androstanediol production that occurs at d21. The more characteristic features of puberty such as the onset of P450scc and P450c17 α expression and an increase in plasma and intratesticular concentrations of androstenedione and testosterone occur at d24, ~3 days later than that in rats (Benton *et al.* 1995, Ge *et al.* 1996). During this time period (from d21 to d24), the murine LCs become more responsive to LH, as demonstrated by the rise in LH-stimulated testosterone production. In contrast, these maturational changes occur in rats at d21 (Benton *et al.* 1995). Our data suggest that the initial differentiation of mouse mesenchymal LCs to progenitor LCs may occur at d24 rather than at d21 as in rats.

The androstanediol data suggest that murine progenitor LCs differentiate into immature LCs by d35, similar to the timing reported in rats (Benton *et al.* 1995). The pubertal decline in androstanediol production and 3 α Hsd expression and corresponding rise in testosterone at d45 are hallmarks of terminal LC differentiation, and indicate that maturation of the LCs from an immature to adult stage occurs at d45 in mice. In contrast, these changes are observed at d56 in rats (Benton *et al.* 1995). Thus, the maturational progression from progenitor to adult LCs in CD1 mice occurs in a compressed developmental window as compared with rats.

In conclusion, our data show that the postnatal development of mouse and rat LCs differs in its onset and pace of progression. Therefore, data on rat LC maturation must be applied with caution to studies in mice. This study complements existing normative data on the patterns of androgen biosynthesis and differentiation in mice to facilitate the assessment of reproductive phenotypes of recombinant mouse models.

Materials and Methods

Animals

CD1 mice were purchased from Charles River Laboratories (Wilmington, MA, USA and Raleigh, NC, USA). Blood and testes were collected after killing by carbon dioxide asphyxiation at 1, 7, 14, 18, 21, 24, 28, 35, 45, 60, 75, 90, and 180 days. The day of birth was designated as d1. All experimental protocols were approved by the Institutional Animal Care and Use Committees of the University of

Massachusetts Medical School (A1619), and Duke University Medical Center (A 433-00-09).

To prepare testicular extracts, the testes were homogenized in 900 μ l TBSG buffer (0.1% gelatin (w/v), 28 mM Trizma HCl, 22 mM Trizma base, 100 mM NaCl, and 15 mM NaN₃). After pelleting cellular debris, the supernatants were collected for hormone analysis.

Isolation of primary LCs

Testes were harvested, decapsulated, dispersed with collagenase (0.05% (w/v), and fractionated in Percoll gradient as described for rat LC isolation (Klinefelter *et al.* 1987, 1993) with minor modifications (Salva *et al.* 2001). The fractionated LCs were purified through a 2.5–10% gradient of BSA (Sigma) to remove contaminating sperm (Salva *et al.* 2001). The purity of each LC preparation, assessed by 3 β HSD staining with 0.4 mM etiocholanolone, was >90% (Payne *et al.* 1980).

In vitro cell culture

Freshly harvested LCs were cultured at 2.5×10^4 cells/ml in serum-free media (phenol red-free DMEM/F12) supplemented with 15 mM HEPES, 26 mM sodium bicarbonate, 0.1% BSA (w/v), 0.0012% gentamicin (w/v), and either 0.07 nM (1 ng/ml) or 7 nM (100 ng/ml) LH. After gentle rotatory agitation for 3 h in a 34 °C water bath at 105 r.p.m. (for PLCs and ILCs) or 85 r.p.m. (for ALCs), the cells were pelleted by centrifugation at 16000 g for 8 min. Conditioned media were collected and stored at –20 °C until hormone assays.

Hormone assays

Testosterone, androstenedione, and androstanediol were measured by RIA in plasma, in conditioned medium from *in vitro* LC cultures, and in supernatants of total testicular homogenates (Coffey *et al.* 1971, Cochran *et al.* 1979, 1981). All samples were assayed in duplicate, and each experimental data point consisted of 3–6 samples. The androstenedione rabbit antiserum (Sigma) is 100% specific for androstenedione (Weinstein *et al.* 1972). Testosterone antibody was obtained from Dr Gordon Niswender (Cochran *et al.* 1981), and androstanediol rabbit antiserum was obtained from Dr Gerald Barbe, University of Western Ontario, London, Canada (Zamecnik *et al.* 1977). Both antibodies are highly specific and have minimal cross-reactivity with other commercial steroids. The lower limit of detection for the androgen assays was 114 pM (33 pg/ml). Intra- and inter-assay coefficients of variation (CV) were 9.04 and 7.76% respectively for testosterone; 6.1 and 5.76% for androstenedione; and 6.98 and 3.97% for androstanediol. LH was assayed by mouse sandwich IRMA at the University of Virginia (UVA) Center for Research in Reproduction Ligand Assay and Analysis Core (NICHD, U54-HD28934). The sensitivity of the LH assay is 5×10^{-3} nM (0.07 ng/ml). The intra-assay CV of 6.0% for the LH assay was determined by taking the average of CV for all controls (in the low, medium, and high range) in that particular assay.

Table 2 Primer sequences for real-time PCR.

Gene name	Sense	Antisense
LHR	5'-TAACCACCATACCAGGGAACGCTT-3'	5'-TAACTCCAGCGAGATTAGCGTCGT-3'
StAR	5'-ACAACCAGGAAGGCTGGAAG-3'	5'-ATGCAGGTGGGGCCGTGTTCA-3'
3 β HSDI	5'-CTCAGTTCCTTAGGCTTCAGCAATTAC-3'	5'-CCAAAGGCAAGATATGATTAGGA-3'
5 α -reductase type I	5'-TCACCTTTGTCTTGGCCTTCCTGT-3'	5'-GTTTCCCCTGGTTTCTCAGATTC-3'
3 α HSD	5'-GGAAACTGTTGGCTGAAGCA-3'	5'-CCAGTCCGGCATCTTACACTT-3'
17 β HSD III	5'-ATTTTACCAGAGAAGACATCT-3'	5'-GGGGTCAGCACCTGAATAATG-3'
3 β HSDVI	5'-AAGCCTCTCAGCACCTCTTG-3'	5'-TGTTTCTTCCATTGGGTTTCA-3'
Actin	5'-ACTTGGCTCAGGAGGAGCAATG-3'	5'-ACAGCTGAGAGGAAATCGTGCG-3'

The inter-assay CV of 12.5% was the mean overall CV for the LH assay in the calendar year of the assay, derived by calculating the mean of all CV for the low, medium, and high-range controls.

Northern blot analysis

To assess androgen biosynthetic capacity, the expression of P450scc and P450c17 α was examined by northern blot analysis. Total testicular RNAs were isolated using TRIzol (Invitrogen). Ten micrograms of RNA were loaded in each lane and electrophoresed in a 1.0% denaturing agarose gel in MOPS, blotted onto nylon membranes (NEN Life Science Products Inc., Boston, MA, USA), and hybridized overnight at 68 °C with 1×10^6 cpm/ml of P450scc- or P450c17 α P32-labeled probes. The membranes were reprobated with a S16 housekeeping gene probe at 42 °C to normalize the target signal. At each age, northern blot analyses were performed with three different RNA isolates from whole testes. Band intensities were quantified by ImageJ Software, normalized to S16, and results were expressed as mean $\pm$ S.E.M.

Quantitative RT-PCR

RNA was extracted from freshly isolated LCs using TRIzol (Invitrogen), and was then treated with DNase and used for cDNA synthesis using the High Capacity cDNA RT kit (Applied Biosystem, Foster, CA, USA). cDNA was used for QPCR amplification using the Mastercycler Realplex2 (Eppendorf, Westbury, NY, USA). For all experiments, murine β -actin was amplified as an internal quality control. The QPCR was carried out in 20 μ l volume using the QuantiTect SYBR Green PCR kit (Qiagen). Each reaction mixture contained $1 \times$ reaction buffer, 2.5 mM MgCl₂, 200 μ M dNTPs, 150–400 nM of the specific primer, and HotStarTaq DNA polymerase. After incubation at 95 °C for 15 min and 45 cycles of 95 °C for 20–30 s, 52–56 °C for 20–30 s, and 72 °C for 20–45 s, the relative amount of the initial mRNA copies was determined by calculating the difference (ΔC_t) in threshold cycle (C_t) value between the target gene and actin, which was then interpreted as $2^{-\Delta C_t}$ to represent the target gene concentration. β -Actin was selected as the housekeeping gene after screening of several housekeeping genes and finding that β -actin expression did not vary significantly from d21 onwards in isolated LCs (O'Shaughnessy *et al.* 2002). The reaction efficiency of each gene was calculated according to $E = 10^{(-1/\text{slope})}$, and it ranged from 1.95 to 2.10 (Rasmussen 2001). The mRNA expression of each transcript was calculated as the ratio of the target gene to β -actin using

$2^{-(\text{target } C_t - \text{actin } C_t)}$, which is comparable with the Livak method (Livak & Schmittgen 2001). The real-time PCR analysis was repeated at least three times for each gene with different pools of primary LCs. The data represent the mean $\pm$ S.E.M. of at least three RT-PCR analyses. All primers except 3 β HSD VI were designed to produce an amplicon spanning at least one intron–exon boundary (see Table 2 for primer sequences).

Immunohistochemistry

Testes from a minimum of three mice at d21 and d24 were fixed in Bouin's fixative overnight, and then embedded in paraffin for cutting of 5- μ m sections. For immunohistochemistry, the sections were blocked for 1 h in 1.5% normal goat serum, and then incubated overnight at 4 °C with the primary antibody at a concentration of 1:2000 for both anti-P450c17 (Dr Dale Buchanan Hales, University of Illinois, Chicago, IL, USA) and anti-P450scc (Research Diagnostics, Flanders, NJ, USA). The sections were incubated with biotinylated anti-rabbit IgG secondary antibody and then with the avidin–biotin immunoperoxidase system (Vectastain ABC kit, Vector Laboratories, Inc., Burlingame, CA, USA) and 3,3'-diaminobenzidine tetrachloride (Boehringer Mannheim). The sections were counterstained with hematoxylin. Alternate sections were processed with PBS instead of the primary antibody for a negative primary antibody control. P450scc and P450c17 staining of sections from d21 and d24 mice were compared by counting 500 cells in random sections.

Statistical analysis

All computations and data analyses were performed using the mixed model of ANOVA in SAS (SAS 1997). Androgen and LH concentrations below the lower limits of the assays were considered undetectable, and were assigned values of 7×10^{-4} nM (0.01 ng/ml) for statistical analysis. Differences were regarded as significant if $P < 0.05$.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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