Asian Journal of Andrology (2013) 15, 685–691
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ORIGINAL ARTICLE

Effects of luteinizing hormone and androgen on the development of rat progenitor Leydig cells *in vitro* and *in vivo*

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Progenitor Leydig cells are derived from stem cells. The proliferation and differentiation of progenitor Leydig cells significantly contributes to Leydig cell number during puberty. However, the regulation of these processes remains unclear. The objective of the present study was to determine whether luteinizing hormone (LH) or androgen contributes to the proliferation and differentiation of progenitor Leydig cells. Fourteen-day-old male Sprague–Dawley rats were treated for 7 days with NalGlu, which is a gonadotropin-releasing hormone antagonist, to reduce the secretion of LH in the pituitary and thus, androgen in the testis. Rats were co-administered with LH or 7α-methyl-nortestosterone (MENT), which is an androgen resistant to metabolism by 5α-reductase 1 in progenitor Leydig cells, and the subsequent effects of LH or androgen were measured. ^{3}H-Thymidine was also intravenously injected into rats to study thymidine incorporation in progenitor Leydig cells. Progenitor Leydig cells were examined. NalGlu administration reduced progenitor Leydig cell proliferation by 83%. In addition, LH or MENT treatment restored Leydig cell proliferative capacity to 73% or 50% of control, respectively. The messenger RNA levels of proliferation-related genes were measured using real-time PCR. The expression levels of *Igf1*, *Lifr*, *Pdgfra*, *Bcl2*, *Ccnd3* and *Pcna* were upregulated by MENT, and those of *Pdgfra*, *Ccnd3* and *Pcna* were upregulated by LH. Both LH and MENT stimulated the differentiation of progenitor Leydig cells *in vitro*. We concluded that both LH and MENT were involved in regulating the development of progenitor Leydig cells.

Asian Journal of Andrology (2013) **15,** 685–691; doi:10.1038/aja.2013.55; published online 24 June 2013

Keywords: luteinizing hormone; progenitor Leydig cell; proliferation; testosterone

INTRODUCTION

Testosterone, which is secreted by Leydig cells in the testis, is the main androgen in circulation. The postnatal development of adult Leydig cells in the rat can be conceptually divided into three distinct stages: progenitor, immature and adult Leydig cells. The first committed Leydig cell is the progenitor Leydig cell. It is spindle-shaped and expresses the Leydig cell lineage biomarker 3β-hydroxysteroid dehydrogenase 1 (3βHSD1) and appears in the interstitium around postnatal days 11–14.[1,2] During the first week after the appearance of progenitor Leydig cells, these cells rapidly divide to increase Leydig cell numbers[3] and then differentiate into immature Leydig cells at day 28 postpartum.[4]

However, the hormonal control of Leydig cell proliferation and differentiation is less well understood in the progenitor Leydig cell compared to the immature or adult Leydig cell, but several factors are known to be involved. These factors include luteinizing hormone (LH), insulin-like growth factor-1 (IGF-1), transforming growth factor α, transforming growth factor β, interleukin 1, thyroid hormone, anti-mullerian hormone and androgen.[4,5] Progenitor Leydig cells are less sensitive to LH stimulation for its steroidogenesis compared to that of androgen,[6] perhaps due to the higher number of androgen receptors compared to the LH receptors (LHCGR) expressed by the rat progenitor Leydig cell.[6] Androgen may also affect the development of progenitor Leydig cells because testicularly feminized mouse testes exhibit a lower Leydig cell number, and androgen may effect steroidogenesis due to androgen insensitivity.[7,8] However, the method by which LH and androgen regulate the development of progenitor Leydig cells still remains unclear. The objective of the present study was to investigate the effects of LH and androgen on the development of progenitor Leydig cells *in vivo* and *in vitro*.

MATERIALS AND METHODS

Materials

NalGlu (NAL [Ac-D2Nal1,D4-ClPhe2,D3Pal3,Arg5,DGlu6(AA),DAla10], a synthetic gonadotropin-releasing hormone antagonist that is used to suppress endogenous LH,[9] was provided by Dr Jean Rivier (Salk Institute, San Diego, CA, USA). Hyamine hydroxide was purchased from ICN Radiochemicals (Irvine, CA, USA). ^{3}H-Thymidine was purchased from

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Received: 2 January 2013; Revised: 16 February 2013; Accepted: 8 April 2013; Published online: 24 June 2013

DuPont-New England Nuclear (Boston, MA, USA). LH was a gift from NIDDK. Because progenitor Leydig cells in the 14-day-old rat testis exhibited high levels of 5α-reductase 1 and 3α-hydroxysteroid dehydrogenase activities,[10] which readily metabolized testosterone into weak androgen androsterone, a synthetic 5α-reductase-resistant androgen 7α-methyl-19-nortestosterone (MENT), which is resistant to 5α-reductase 1,[10,11] was used. MENT was 10 times more potent than testosterone in binding to the androgen receptor.[10,11] MENT was kindly provided by the Upjohn Company (Kalamazoo, MI, USA). Sprague–Dawley rats (dams with litters of male pups) were purchased from Charles River Laboratories (Wilmington, MA, USA). The animal protocols were approved by the Institutional Animal Care and Use Committee of the Rockefeller University and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Methods

Animals and treatment. Male pups from postnatal day 14, a time in which progenitor Leydig cells are formed,[4] were randomly divided into four groups with 40 animals per group. Three sets of experiments were performed. To suppress the endogenous secretion of LH and androgen, NAL was administered daily *via* intraperitoneal injection. The rats were treated with hormones for 7 days as follows: (i) control (CON) rats received an injection of 8% mannitol (vehicle); (ii) rats in the second group (NAL group) received intraperitoneal injections of NAL (which was dissolved in 8% mannitol) at a dose of 10 μg day^{-1}; (iii) rats in the third group (NAL+LH group) received injections of NAL plus 5 μg LH per day; and (iv) rats in the fourth group (NAL+MENT group) received injections of NAL plus 25 μg day^{-1} MENT. Doses of LH or MENT were adopted according to previously reported observations.[11] Each hormone was dissolved in 8% mannitol and injected in the rats (0.1 ml per rat) twice per day. The treatment began on day 14 postpartum, and the animals were sacrificed on day 21 by asphyxiation with CO_2.

In vivo *^{3}H-thymidine incorporation.* After 7 days of hormonal treatment, half of the rats in each group received 1 μCi g^{-1} body weight ^{3}H-thymidine by intraperitoneal injection. The rats were euthanized by CO_2 exactly 2 h later. The testes were removed and grouped according to the treatment for the isolation of progenitor Leydig cells.

Cell isolation. Both ^{3}H-thymidine-treated and untreated rats were used for the isolation of progenitor Leydig cells. The isolation procedure for the progenitor Leydig cells was performed as previously described.[12] Briefly, testes from five 21-day-old rats after hormonal treatment were removed. Decapsulated testes were dispersed with 0.25 mg ml^{-1} collagenase (collagenase-D; Boehringer Mannheim Biochemicals, Indianapolis, IN, USA) in medium 199 for 10 min at 34 °C with shaking. The separated cells were filtered through two layers of nylon mesh, centrifuged at 250 *g* and resuspended in 55% isotonic Percoll. Followed by density gradient centrifugation at 25 000*g* for 45 min at 4 °C, the progenitor Leydig cell fraction was collected between densities of 1.064 and 1.070 g ml^{-1}. The cells were washed with Hank's buffered salt solution, centrifuged at 250 *g* and then resuspended in phenol red-free Dulbecco's modified Eagle medium: Nutrient mixture F-12 (DMEM-Ham's F-12) medium (D-2906; Sigma Chemical Co., St Louis, MO, USA) supplemented with 1 mg ml^{-1} bovine serum albumin. Three replicates of the isolation were performed. The purity of the progenitor Leydig cells was determined by performing immunohistochemical staining of 3βHSD1, using 0.4 mmol l^{-1} etiocholanolone as substrate and NAD+ as cofactor according to a previously described method.[13] Enrichment of the progenitor Leydig cells was typically up to 95% purity.

Ex vivo *^{3}H-thymidine incorporation.* Progenitor Leydig cells (0.2×10^{6} cells) isolated from rats after hormonal treatment for 7 days (at postnatal day 21) without ^{3}H-thymidine treatment were incubated in DMEM-Ham's F-12 medium containing 1 μCi ml^{-1} ^{3}H-thymidine for 2 h. The media was decanted from the cells after centrifugation.

Measurement of radioactivity. Triplicate aliquots of 1×10^{6} cells from ^{3}H-thymidine-treated rats in each group were dispensed into 1.5 ml microcentrifuge tubes. Both cells from the *in vivo* and *in vitro* treatment of ^{3}H-thymidine were pelleted after centrifugation at 250 *g* for 10 min and the cells were washed twice using phosphate buffered saline. The cells were dissolved with 0.15 ml hyamine hydroxide, and radioactivity was measured in a liquid scintillation counter as previously described.[5]

Histological analysis. Five testes were randomly collected from each of the four groups and perfused after punching three holes in each testis using a needle. Subsequently, the testes were fixed by immersion with 2.5% glutaraldehyde, 1% acrolein in 0.1 mol l^{-1} sym-collidine buffer, 1% osmium for postfixation and 1.5% potassium ferricyanide, and were then embedded in Epon-araldite resin. Testicular sections were cut at a thickness of 3 μm and stained with 1% toluidine blue to identify mesenchymal cells and progenitor Leydig cells, as previously described.[3] Mesenchymal cells are characterized as fusiform cells and do not touch the basal lamina of the seminiferous tubule or the vascular endothelium. Progenitor Leydig cells were identified by their abundant, darkly stained cytoplasm and irregular nuclei. The morphology of progenitor Leydig cells was oval-like or round. Other interstitial cell types including macrophages, pericytes and endothelial cells were identified according to a previously published method.[3] The number of progenitor Leydig cells, mesenchymal cells and other interstitial cells were quantified, according to the fractionator technique.[3,11] Approximately 10 sections were sampled from each testis. The total number of interstitial cells was calculated by multiplying the number of interstitial cells quantified in a known fraction of the testis by the inverse of the sampling probability. The percentage of each cell type of the total number of interstitial cells was calculated for statistical analysis.[3,11]

Cell culture and in vitro *hormonal treatment.* Progenitor Leydig cells obtained from normal 14-day-old rat testes were also purified according to the previously described method. Progenitor Leydig cells were cultured for 48 h in phenol red-free medium (DMEM-Ham's F12) supplemented with 1 mg ml^{-1} bovine serum albumin, 1 mg ml^{-1} bovine lipoprotein and 25 m mol l^{-1} HEPES (pH 7.2) in a 34 °C, 5% O_2 in a 5% CO_2 humidified incubator. In the animal groups that received *in vitro* hormonal treatment, Leydig cells were first cultured for 24 h in hormone-free medium. The media were then removed and replaced with fresh medium containing either 1 ng ml^{-1} ovine LH; 50 n mol l^{-1} MENT or 1 ng ml^{-1} LH plus 50 n mol l^{-1} MENT *in vitro* for 7 days. The concentrations of LH and MENT used in the present experiment were selected based on their effective action on the proliferation of progenitor Leydig cells, as described in our previous study.[5] The medium was changed daily and collected for the measurement of androsterone, which is a primary androgen produced by progenitor Leydig cells,[10] and testosterone.

Radioimmunoassay for androsterone and testosterone. The radioimmunoassay for androsterone and testosterone was performed as previously described,[10] using a specific antibody against either androsterone or testosterone. Androsterone was measured because progenitor Leydig cells secrete this steroid at approximately 80% of total androgen levels.[10] The intrassay and interassay coefficients of variation were within 15% for radioimmunoassay.

Quantitative real-time PCR analysis. Total RNAs were extracted from the isolated progenitor Leydig cells (1×10^6 cells) in Trizol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The RNAs were reverse transcribed into cDNAs in the presence of random primers and the M-MLV reverse transcriptase (Promega Biosciences, Inc., San Luis Obispo, CA, USA). Quantitative PCR in the presence of SYBR green was performed on an ABI 7700 system (PE Applied Biosystems, Carlsbad, CA, USA). Messenger RNA measurements were normalized against an internal control, which was the ribosomal protein S16 (*Rps16*). All control Cycle threshold (C_t) values were corrected by the median difference between the samples and *Rps16*. The C_t values for *Igf1*, *Lifr*, *Pdgfra*, *Bcl2*, *Ccnd3*, *Pcna*, *Nr5a1*, *Hsd3b1*, *Kit* and *Rps16* were 22, 17, 16, 20, 19, 20, 17, 19, 21 and 16, respectively. The primer sequences used and full gene names are listed in **Table 1**.

Statistical analysis. Data were expressed as the mean±s.e.m. A one-way ANOVA with Tukey's *post hoc* analysis was used to determine differences between two groups when more than three groups were compared using GraphPad (Version 5; GraphPad Software, Inc., San Diego, CA, USA). Differences were regarded as statistically significant at $P<0.05$. All of the measurements were repeated at least three times.

RESULTS

Testis weights, morphology and percentage of interstitial cells after hormonal treatment

As shown in **Figure 1**, NAL treatment significantly decreased the testis weight when compared to control ($P<0.001$). Administration of LH or MENT in NAL-treated rats partially restored the loss of testis weights. The progenitor Leydig cells were oval-shaped with abundant cytoplasm in the testis of the control rat (**Figure 2**). Mesenchymal cells (**Figure 2a**) were spindle-shaped cells located in the central regions of the interstitial space with little cytoplasm and did not touch the basal lamina of the seminiferous tubule or the vascular endothelium. Other cells included endothelial cells, macrophages and pericytes.

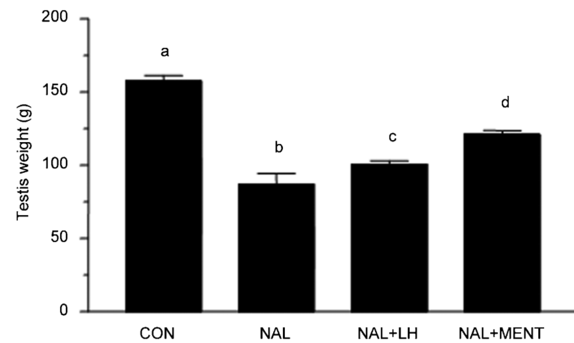

Figure 1 Effects of hormonal treatment on the testis weights of rats. Male pups were treated without (CON) or with NalGlu (NAL), NalGlu plus LH (NAL+LH) and NalGlu plus MENT (NAL+MENT) from postnatal days 14 to 21 (mean±s.e.m., $n=10$). The identical letter indicates that there was no significant difference between two groups at $P<0.05$. LH, luteinizing hormone; MENT, 7α-methyl-nortestosterone.

The average number of cells per testis 7 days after hormonal treatment is shown on **Table 2**. After the administration of NAL, few progenitor Leydig cells were observed, and the mesenchymal cells became the major cell type in the interstitial area (**Figure 2b**). Moreover, treatment with NAL plus LH or NAL plus MENT partially restored the number of progenitor Leydig cells in the interstitial area (**Figures 2c and d and 3**). The percentage of progenitor Leydig cells, mesenchymal cells and other interstitial cells and their absolute numbers were further quantitatively calculated. NAL treatment has been shown to increase the percentage and absolute number of mesenchymal cells and decrease the number of progenitor Leydig cells, while administration with LH or MENT in NAL-treated rats partially restored the percentage and absolute number of progenitor Leydig cells of total interstitial cells (**Figure 3**). The total number of interstitial cells was not affected in all groups.

Table 1 Primer sequences and PCR product sizes

Gene name	*Gene symbol*	*Primer sequence*	*PCR size (bp)*
Insulin-like growth factor 1	*Igf1*	Forward: 5′-ACTCTGCTTGCTCACCTTTACC-3′ Reverse: 5′-TCATCCACAATGCCCGTC-3′	174
Leukemia inhibitory factor receptor	*Lifr*	Forward: 5′-GCTGACTTCTCGACCTCCA-3′ Reverse: 5′-GGTATTACGCGTCCAGGA-3′	310
Platelet-derived growth factor receptor α peptide	*Pdgfra*	Forward: 5′-ACCTTGCACAATAACGGGAG-3′ Reverse: 5′-CAGTTTGATGGACGGGAGTT-3′	336
B-cell lymphoma 2	*Bcl2*	Forward: 5′-AGCGTCAACAGGGAGATGTC-3′ Reverse: 5′-TATGCACCCAGAGTGATGCA-3′	204
G1/S-specific cyclin-D3	*Ccnd3*	Forward: 5′-AGGCACTGGTCAAAAAGCAT-3′ Reverse: 5′-GTCCACTTCAGTGCCTGTGA-3′	191
Proliferating cell nuclear antigen	*Pcna*	Forward: 5′-GCCCTCAAAGACCTCATCAA-3′ Reverse: 5′-TCTGGGATTCCAAGTTGCTC-3′	338
Steroidogenic factor 1	*Nr5a1*	Forward: 5′-CAGAGCTGCAAAATCGACAA-3′ Reverse: 5′-CCCGAATCTGTGCTTTCTTC-3′	186
3β-hydroxysteroid dehydrogenase I	*Hsd3b1*	Forward: 5′-CCCTGCTCTACTGGCTTGC-3′ Reverse: 5′-TCTGCTTGGCTTCCTCCC-3′	178
Kit	*Kit*	Forward: 5′-GGGGATCATTGTGATGGT-3′ Reverse: 5′-GACATTAGGGCCTCCCTTT-3′	308
Ribosomal protein S16	*Rps16*	Forward: 5′-AAGTCTTCGGACGCAAGAAA-3′ Reverse: 5′-TTGCCCAGAAGCAGAACAG-3′	148

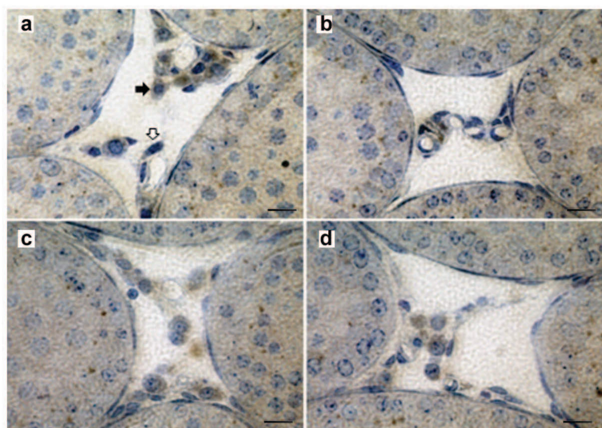

Figure 2 Effects of hormonal treatment on rat interstitial morphology. There were numerous progenitor Leydig cells (indicated by the black arrow) and putative mesenchymal-like mesenchymal cells (indicated by the white arrow) in the control group (**a**). Few progenitor Leydig cells were detected (**b**) in the testicular interstitial areas after NalGlu (NAL) treatment. The partially restored interstitial histology presented in the NAL+LH (**c**) and NAL+MENT (**d**) groups (scale bar=10 μm). LH, luteinizing hormone; MENT, 7α-methyl-nortestosterone.

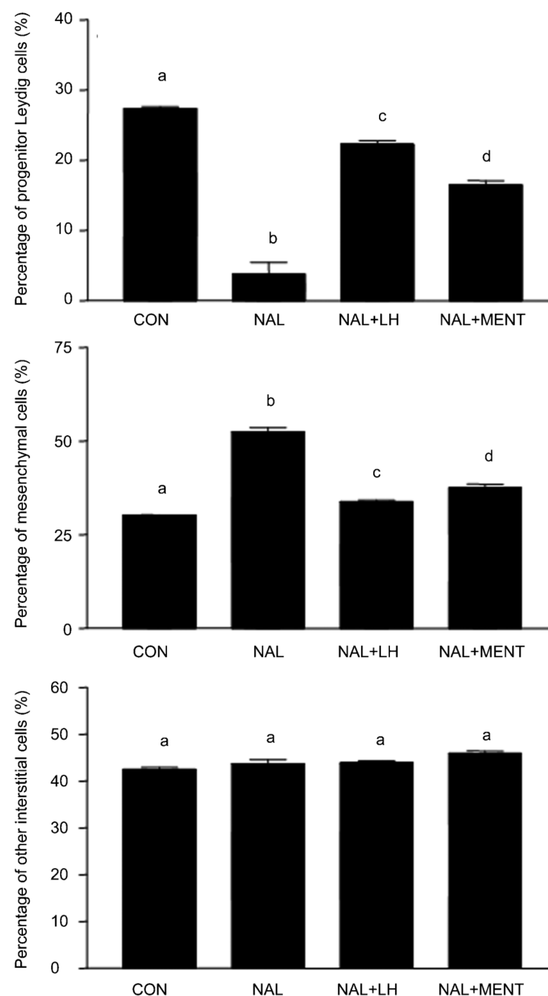

Figure 3 The percentage of PLCs, mesenchymal-like MCs and other interstitial cells of total interstitial cells. Male pups were treated without (CON) or with NalGlu (NAL), NalGlu plus LH (NAL+LH) and NalGlu plus MENT (NAL+MENT) from postnatal days 14 to 21 (mean±s.e.m., n=5). An identical letter indicates that there was no significant difference between two groups at $P<0.05$. LH, luteinizing hormone; MC, mesenchymal cell; MENT, 7α-methyl-nortestosterone; PLC, progenitor Leydig cell.

In vivo and *ex vivo* thymidine incorporation of isolated progenitor Leydig cells

Thymidine incorporation by progenitor Leydig cells was measured to examine the proliferative capacity of progenitor Leydig cells *in vivo* and *in vitro*. As shown in **Figure 4**, thymidine incorporation in progenitor Leydig cells was significantly reduced to only 17% of control levels after NAL treatment as shown in both *in vivo* (**Figure 4a**) and *ex vitro* (**Figure 4b**) thymidine incorporation studies. Compared to NAL treatment, LH or MENT administration in NAL-treated rats resulted in higher thymidine incorporation levels, which were 73% and 50% of control levels, respectively. These data indicated that LH and MENT were required for the maintenance of the proliferative capacity of progenitor Leydig cells.

Messenger RNA levels of proliferation-related genes in progenitor Leydig cells

We isolated progenitor Leydig cells from each treatment group and measured the mRNA levels of several genes that were expected to have roles in proliferation or differentiation. As shown in **Figure 5**, NAL treatment significantly reduced mRNA levels of proliferating cell nuclear antigen (*Pcna*), B-cell lymphoma 2 (*Bcl2*) and cyclin D3 (*Ccnd3*), which are genes that encode proteins that are involved in proliferation. NAL also significantly downregulated the expression levels of the growth factor-related genes *Igf1*, leukemia inhibitory factor receptor (*Lifr*) and platelet-derived growth factor receptor α polypeptide (*Pdgfra*).

Table 2 Absolute cell number per testis (millions) after hormonal treatment

Treatment	*Leydig cell*	*Mesenchymal cell*	*Other cells*	*Total cell*
Control	2.708±0.202[a]	1.151±0.103[a]	1.105±0.086[a]	4.964±0.243[a]
NAL	0.226±0.071[b]	2.762±0.163[b]	1.381±0.103[a]	4.369±0.219[a]
NAL+LH	1.941±0.090[c]	1.473±0.117[a]	1.381±0.073[a]	4.795±0.217[a]
NAL+MENT	1.715±0.291[c]	1.243±0.092[a]	1.197±0.086[a]	4.155±0.375[a]

Abbreviations: LH, luteinizing hormone; MENT, 7α-methyl-nortestosterone.
Values are expressed as the mean±s.e.m. Within each column, numbers that were not significantly different ($P>0.05$) share the same letter.

However, there was no change in *Kit* mRNA levels after hormonal treatment. The expression of *Nr5a1* (encoding steroidogenic factor 1) and *Hsd3b1* (encoding 3βHSD1), which were expected in differentiating cells, was also downregulated. LH or MENT administration in NAL-treated rats restored the expression of the proliferative biomarkers *Pcna* and *Ccnd3*. However, LH and MENT regulated growth factor-related gene expression differently. LH increased the expression of *Pdgfra*, but did not affect the level of *Igf1*, *Bcl2* and *Lifr*, while MENT increased the expressions of *Igf1*, *Pdgfra*, *Lifr and Bcl2*. As expected, LH significantly increased the expression levels of differentiation-related genes, including *Nr5a1* and *Hsd3b1*. MENT also slightly but significantly increased *Nr5a1* expression but did not affect *Hsd3b1*. These data suggested that LH and MENT regulated different sets of genes to stimulate progenitor Leydig cell proliferation and/or differentiation.

LH and MENT stimulate the differentiation of progenitor Leydig cells

We isolated progenitor Leydig cells and cultured them with LH and MENT alone or in combination for 7 days. As shown in **Figure 6**, LH

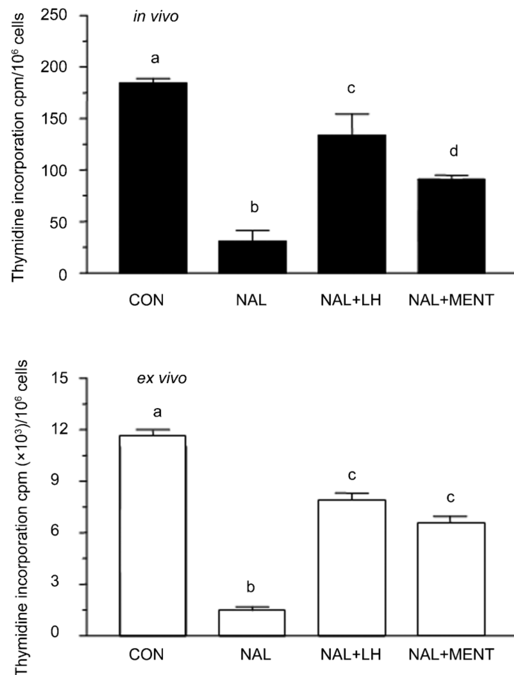

Figure 4 Thymidine incorporation in isolated progenitor Leydig cells after hormonal treatment. Male pups were treated without (CON) or with NalGlu (NAL), NalGlu plus LH (NAL+LH) and NalGlu plus MENT (NAL+MENT) from postnatal days 14 to 21 (mean±s.e.m., n=3). An identical letter indicates that there was no significant difference between two groups at P<0.05. LH, luteinizing hormone; MENT, 7α-methyl-nortestosterone.

and MENT significantly increased testosterone production, and their combination was synergistic.

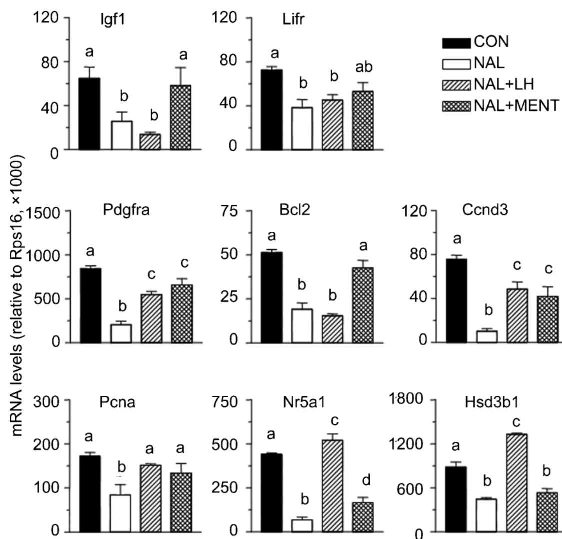

Figure 5 Messenger RNA levels of the target genes. Male pups were treated without (CON) or with NalGlu (NAL), NalGlu plus LH (NAL+LH) and NalGlu plus MENT (NAL+MENT) from postnatal days 14 to 21. The *Igf1*, *Lifr*, *Pdgfra*, *Bcl2*, *Ccnd3*, *Pcna*, *Nr5a1* and *Hsd3b1* expression levels were measured in isolated progenitor Leydig cells. The data are shown as the mean±s.e.m., n=3 preparations. An identical letter indicates that there was no significant difference between two groups at P<0.05. LH, luteinizing hormone; MENT, 7α-methyl-nortestosterone.

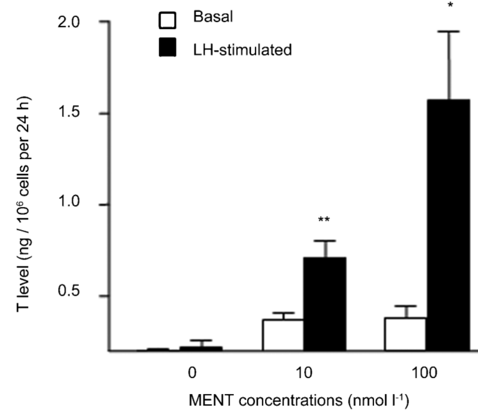

Figure 6 The effects of LH and MENT alone or in combination on testosterone production of progenitor Leydig cells. The data are shown as the mean±s.e.m., n=4 preparations. *P<0.05 or **P<0.01, compared to control (no MENT treatment). LH, luteinizing hormone; MENT, 7α-methyl-nortestosterone.

DISCUSSION

This *in vivo* study showed that LH and androgen stimulated the proliferation of progenitor Leydig cells in NAL-castrated testes and also regulated different sets of growth factor-related genes. We also demonstrated that *in vitro* LH and MENT alone could stimulate the differentiation of progenitor Leydig cells.

Previous studies have shown that factors other than LH have roles in the proliferation and differentiation of cells in the Leydig cell lineage. It has been shown that proliferation of precursor cells in the Leydig cell lineage occurs in the absence of LH.[14] One of these factors is prolactin. Prolactin receptors are expressed on progenitor Leydig cells in the prepubertal rat testis and it was found that prolactin stimulated their proliferation.[15] Androgen could be another factor. The hypothesis that androgens facilitate the proliferation of Leydig cell precursors is based on the evidence that androgen receptors outnumber LH receptors in progenitor Leydig cells.[6]

Gonadotropin-releasing hormone antagonists have been used to suppress endogenous LH and androgen.[16] Treatment with the gonadotropin-releasing hormone antagonist NAL has been demonstrated to reduce testis weight and pituitary LH secretion and thus, lower androgen levels.[16] Gonadotropin-releasing hormone completely suppresses circulating LH levels, which in turn decreases the cytoplasmic mass of adult Leydig cells. This is associated with an almost undetectable testosterone level (only 6% of control).[17] However, some concerns have arisen regarding the mechanism of gonadotropin-releasing hormone antagonist because rat Leydig cells express high affinity gonadotropin-releasing hormone receptors.[18] However, the blockade of these gonadotropin-releasing hormone receptors with a gonadotropin-releasing hormone antagonist had no direct effect on rat Leydig cell function *in vivo*,[19] which may indicate that gonadotropin-releasing hormone antagonists suppress circulatory levels of LH and androgen. In a previous study, NAL, a gonadotropin-releasing hormone antagonist, was used to distinguish the effects of exogenous LH and androgen on the initial differentiation of Leydig cells from progenitor Leydig cells.[11] Treatment with NAL induced significant reductions in testis weight (also shown in **Figure 1** of this study), testosterone production and the cytoplasmic mass of progenitor Leydig cells, and these parameters were partially recovered after co-administration with LH or testosterone.[11] The partial restoration of testis weight and cytoplasmic mass of progenitor Leydig cells by MENT (a synthetic androgen) was observed in the present study (**Figures 1**

and 2). In this study, we also demonstrated that NAL treatment significantly reduced the proliferative capacity of progenitor Leydig cells (**Figure 4**).

LH is essential for Leydig cell function because it is the principle stimulating hormone of androgen production by adult Leydig cells.[4] Our present study showed that LH maintained the proliferative capacity of progenitor Leydig cells at 73% of control levels in the NAL-treated testis (**Figure 4**).

When immature rats were treated with gonadotropin-releasing hormone antagonist and testosterone simultaneously, the morphology of Leydig cell precursors and levels of Leydig cell-specific mRNA levels for *Cyp17a1* and *Lhcgr* were similar to controls.[20] In addition, androgen was likely to be required for the differentiation of progenitor Leydig cells.[12] In the present study, we demonstrated that *in vitro* culture of MENT significantly increased total androgen production. Although androgen may exert autocrine regulation of progenitor Leydig cell differentiation (**Figure 6**), we previously demonstrated that androgen did not stimulate progenitor Leydig cell proliferation but stimulated immature Leydig cell proliferation *in vitro*.[5] Xu *et al.*[21] also showed that androgen receptors expressed on Leydig cells were not required for Leydig cell proliferation because the number of Leydig cells was normal in Leydig cell-specific androgen acceptor knockout (L-AR-/y) mice. Furthermore, our present study also showed that MENT partially maintained progenitor Leydig cell proliferation *in vivo* (**Figure 3**), indicating that this action may exert indirectly *via* other testicular cell types. In addition to Leydig cells in the testis, Sertoli cells and myoid cells also express androgen receptors.[22] The *in vivo* effects of androgens on Leydig cell number are potentially mediated *via* androgen receptors in Sertoli cells because a significant reduction in mouse Leydig cell number was observed in Sertoli cell-specific knockout androgen receptor mice.[23] One downstream target of androgen receptors in Sertoli Cells is PDGF-A because PDGF-A mRNA (Pdgfa) levels in these knockout mice were significantly reduced.[23] Moreover, the myoid cell-specific knockout of androgen receptors did not appear to affect Leydig cell function.[24]

We found that LH and androgen regulated different sets of genes that were associated with progenitor Leydig cell proliferation and differentiation. LH significantly contributed to the mRNA levels of platelet-derived growth factor receptor α peptide (*Pdgfra*) and cyclin D3 (*Ccnd3*). Platelet-derived growth factor α is required for stimulation of the proliferation of stem and progenitor Leydig cells.[25,26] Adult Leydig cells were also completely absent in *Pdgfra*-deficient mice,[26] and thus, this growth factor may be essential for the proper development of adult Leydig cells. Cyclin D3, which is a known mediator of cell proliferation, regulates the checkpoint in DNA synthesis and transition of the phase from G1 into S in the cell cycle.[27] Platelet-derived growth factor has been shown to regulate cell cycle progress by inducing *Ccnd3* expression.[28,29] Thus, the observed low expression of *Pdgfra* after NAL treatment is consistent with the observed low expression of *Ccnd3*.

MENT treatment increased the mRNA levels of *Pdgfra*, *Igf1*, *Bcl2*, *Ccnd3* and *Pcna* (**Figure 5**). IGF-I is required for progenitor Leydig cell proliferation and it has been reported to stimulate DNA synthesis in progenitor Leydig cells *in vitro*.[5] The IGF-I knockout mouse testis has only one-sixth the number of Leydig cells present in age-matched wild-type testis,[30] most likely as the result of a reduced proliferation of progenitor Leydig cells.[31] Leukemia inhibitory factor receptor (*Lifr*) is present in the Leydig cell lineage, including mesenchymal cells.[25,32] Leukemia inhibitory factor can maintain stem cell self-renewal in many types of stem cells,[33] including mesenchymal cells.[25]

Proliferating cell nuclear antigen, a cell-cycle marker protein, is an essential component for eukaryotic chromosomal DNA replication and repair.[34] Our results showed that decreased *Ccnd3* and *Pcna* mRNA levels coincided with the decreased proliferative capacity of progenitor Leydig cells, as assessed by the reduction in thymidine incorporation into the cell (**Figure 3**), and the decreased number of progenitor Leydig cells (**Figure 4**) after NAL treatment. Bcl2 is a negative regulator of cell survival and apoptosis[35] and is present in Leydig cell precursor cells but not in terminally differentiated adult Leydig cells.[36] Our observation that *Bcl2* expression was maintained by androgen in NAL-treated animals (**Figure 5**) suggests that androgen is required for the suppression of apoptosis in progenitor Leydig cells. BCL2 and BAK protein, a pro-apoptotic member of the Bcl-2 gene family, are involved in initiating apoptosis.

In addition, we examined the mRNA levels of steroidogenic factor 1 (*Nr5a1*) and 3βSHD1 (*Hsd3b1*). Steroidogenic factor 1 was identified as a tissue-specific transcriptional regulator of cytochrome P450 steroid hydroxylases in Leydig cells[37] and 3βHSD1 is known as a Leydig cell-specific mRNA transcript. These transcript levels were decreased by NAL treatment. Furthermore, LH, but not MENT treatment, resulted in higher levels of *Nr5a1* and *Hsd3b1* mRNA compared to the untreated groups. It is important to identify distinct functions of LH and androgen, and it is likely that LH administration induced androgen production in NAL-treated rats. However, androgen secreted from 14-day-old rat testis was mainly a weak androgen androsterone,[10] which had nearly no androgen activity. We observed that LH selectively induced *Nr5a1* and *Hsd3b1* mRNAs, while androgen selectively induced *Igf1* and *Bcl2* mRNAs, suggesting that these two hormones act on distinct signaling pathways.

In conclusion, the present study showed that suppression of endogenous LH and androgen resulted in a reduced proliferation of progenitor Leydig cells and a reduced expression of several genes. Our results support the conclusion that androgen contributes to signaling pathways mediated by several growth factors and suppresses apoptosis and that LH induces progenitor Leydig cell differentiation.

AUTHOR CONTRIBUTIONS

YX, RG were responsible for the concept and framework of the paper. JG, XM, CW, YG, QL, and YZ participated in collecting and evaluating the data. JG, QL, DOH and RG wrote the paper. YX and RG were responsible for the drafting and final editing. All authors read and approved the final manuscript.

COMPETING FINANCIAL INTERESTS

All authors declare that there are no competing financial interests.

ACKNOWLEDGMENTS

We are grateful to Ms Chantal Sottas for technical assistance. This work was in part supported by the National Nature Science Foundation of China (nos. 30871434 and 31171425 to RS Ge, no. 30900846 to CQ Wang and no. 81200430 to YF Zhang).

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