The development of rat Leydig cell progenitors *in vitro*: how essential is luteinising hormone?

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Abstract

Luteinising hormone (LH) appears to be important for the establishment of the adult-type Leydig cell population. The role of LH in the initial steps of stem Leydig cell/precursor cell differentiation is less clear. The aim of the present study was to elucidate the role of LH in the differentiation of spindle-shaped mesenchymal-like cells into Leydig cell progenitors. Interstitial cells were isolated from rat testes at three different ages reflecting different phases in development, and cultured in the presence of increasing concentrations of LH (ranging from 0·01 to 10 ng/ml). Cells were isolated from 10-day-old rats when stem Leydig cells/precursor cells are abundant; 13-day-old rats when the first 3β-hydroxysteroid dehydrogenase (3β-HSD)-positive Leydig cell progenitors have developed in the strain of rats used in this study; and 18-day-old rats just prior to the wave of progenitor proliferation. Immunohistochemistry revealed that before stem Leydig cells differentiate into progenitor cells, they acquire functional LH receptors and become precursor cells. This was confirmed by an *in vivo* immunohistochemical double-labelling experiment. Addition of LH to the cultures increased the percentage of LH/3β-HSD-labelled Leydig cell progenitors, while the percentage of cells solely expressing the LH receptor decreased. Cell proliferation was negligible, suggesting that the increase in 3β-HSD-positive cells is the result of precursor cell differentiation. When interstitial cells were isolated from 13-day-old rats and to a lesser extent from 10-day-old rats, a small proportion of the precursors could develop into progenitor cells independent of the presence of LH. In conclusion: before Leydig stem cells differentiate into 3β-HSD-positive progenitor cells, they acquire LH receptors and become precursor cells. LH appears to be essential, even at very low doses for the differentiation of these precursor cells into 3β-HSD-positive progenitors, although a subpopulation of precursor cells can develop into progenitors independently of LH.
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Introduction

The main function of Leydig cells in the prepubertal and adult testis is the production of androgens, essential for the progression of spermatogenesis. Synthesis and secretion of androgens by Leydig cells is stimulated by the gonadotrophin luteinising hormone (LH; Ahmad *et al.* 1973, Sharpe 1987). Testicular secretion of androgens depends not only on the activity of the interstitial Leydig cells, but also on the number of these cells present in the testis.

In rats, two defined periods of proliferation and differentiation can be discerned before the final population of Leydig cells in the adult testis is established. The first wave occurs during foetal life and results in a foetal population of Leydig cells. The second wave takes place during the prepubertal period and gives rise to the adult population of Leydig cells (Mendis-Handagama *et al.* 1987, Hardy *et al.* 1989). After birth, the number of foetal-type Leydig cells undergoes some decrease; 50–75% of the original foetal-type Leydig cell population present at birth persists in the adult testis (Kerr & Knell 1988). The second generation of Leydig cells, the so-called adult-type Leydig cells, develops from stem Leydig cells through several steps of differentiation and proliferation during (pre)puberty (Lording & de Kretser 1972, Hardy *et al.* 1989, De Kretser & Kerr 1994, Mendis-Handagama & Ariyaratne 2001, Haider 2004, Ge *et al.* 2006). Spindle-shaped stem Leydig cells of mesenchymal origin, identified by the presence of platelet-derived growth factor (PDGF) receptor-α, leukaemia-inhibiting factor (LIF) receptor and c-kit, and the absence of LH receptors and steroidogenic enzyme expression, are thought to differentiate into 3β-hydroxysteroid dehydrogenase (3β-HSD)-positive Leydig cell progenitors between days 10 and 13 after birth. Subsequently, these cells undergo further proliferation and differentiate in immature adult-type Leydig cells between days 28 and 35 after birth. By that time, the proliferative activity of these cells is decreasing rapidly and the immature Leydig cells develop into mature terminally differentiated adult-type Leydig cells. By the end of puberty, the development of the adult population is

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completed. Each step in this differentiation process is characterised by specific morphological aspects of the developing cells (Vergouwen *et al.* 1991, Mendis-Handagama & Ariyaratne 2001, Haider 2004) and the expression of specific steroidogenic enzymes, such as 3β-HSD, 5α-reductase, cholesterol side chain cleavage ($P450_{scc}$) and 17α-hydroxylase ($P450_{17\alpha}$; Wiebe 1976, O'Shaughnessy *et al.* 2000, 2002, Ge *et al.* 2005).

A considerable number of studies have been performed to investigate the regulation of this complicated developmental process in more detail (Ketelslegers *et al.* 1978, Chemes *et al.* 1985, Teerds *et al.* 1989, Hardy *et al.* 1990). Treatment of hypophysectomised prepubertal rats with highly purified LH has been shown to stimulate both differentiation of stem Leydig cells/precursor cells and proliferation of the newly formed progenitor Leydig cells (Teerds *et al.* 1989). Similarly, treatment of prepubertal boys with the LH analogue human chorionic gonadotrophin (hCG) induced the formation of new Leydig cells through stem cell/precursor cell differentiation (Chemes *et al.* 1985).

Recently, more information has become available about the identity of the stem Leydig cell/precursor cell and the endocrine/paracrine regulation of Leydig cell development, although these studies are sometimes contradictory. One of the first investigations on Leydig cell differentiation *in vitro* showed that the addition of LH alone did not result in the formation of progenitor cells from mesenchymal-like stem cells/precursor cells. In addition to LH, the androgen dihydrotestosterone (DHT) was required for the transition of these cells into Leydig cell progenitors (Hardy *et al.* 1990, 1991). The specific requirement of DHT is intriguing because endogenous androgens are continuously produced by LH-stimulated foetal-type and progenitor Leydig cells. Moreover, the *in vitro* added DHT is thought to be rapidly metabolised and can thus exert its effect for only a brief period of time (Hardy *et al.* 1990). In knockout studies, the role of LH in the initial phases of Leydig cell development was further questioned. In the absence of the Desert hedgehog (*Dhh*) gene (Clark *et al.* 2000) or PDGF-A (Gnessi *et al.* 2000), Leydig cell progenitor formation did not occur, despite plasma LH levels being normal. When gonadotrophin levels were very low or below the detection limit of the RIA used, such as in hypogonadal (hpg) mice (Baker & O'Shaughnessy 2001) or in case of disruption of LH signalling in LH receptor knockout mice (Zhang *et al.* 2001), adult-type Leydig cell development appeared to be severely affected though possibly not completely inhibited. Moreover, Ariyaratne *et al.* (2000) observed, using immunohistochemical techniques, that in progenitor cells the steroidogenic enzymes 3β-HSD, $P450_{scc}$ and $P450_{17\alpha}$ appeared before LH receptors could be detected. The latter study suggested that, like in foetal-type Leydig cells (Majdic *et al.* 1998), the development of the steroidogenic capacity of the Leydig cell progenitors precedes their responsiveness to LH.

In the present study, a different approach was used to obtain more information concerning the factors involved in the formation of Leydig cell progenitors. We hypothesise that spindle-shaped Leydig stem cells (Ge *et al.* 2006) first develop into LH receptor expressing cells (which will be named spindle-shaped precursor cells to discriminate them from stem Leydig cells that do not express LH receptors) before they differentiate into LH receptor and 3β-HSD-positive progenitor cells. The emphasis in this study is on the role of LH in the conversion of LH receptor-positive precursor cells into progenitor cells. In order to investigate this, we have isolated interstitial cells from rat testes at three different ages reflecting different phases in this developmental process: from 10-day-old rats when in the substrain of Wistar rats used in the present study, foetal-type Leydig cells and Leydig stem cells/precursor cells are present but Leydig cell progenitors have not yet developed; from 13-day-old rats when in the substrain of Wistar rats used in this study, the first Leydig cell progenitors have been formed; and from 18-day-old rats just prior to the wave of proliferation of the progenitor cells (Hardy *et al.* 1989). Spindle-shaped precursor cells and progenitor Leydig cells were identified by the presence of 3β-HSD activity and/or LH receptors. LH-stimulated cAMP production was measured as a functional index of the LH receptors.

Materials and Methods

Isolation of cells

Immature Wistar rats were obtained from the Central Animal Facilities of Utrecht University (Utrecht, the Netherlands). For each experiment, groups of 25–30 animals were killed 10, 13 or 18 days after birth by CO_2 asphyxiation and decapitation. Testes were removed and put in HBSS (Gibco, Life Technologies). Subsequently, testes were decapsulated and subjected to enzymatic digestion at 37 °C in a shaking water bath (80 cycles/min), using 5 mg collagenase (type IV, 213 U/mg; Worthington Biochemical Corp., Freehold, NJ, USA) in 20 ml, Hanks buffered salt solution (HBSS). After 12.5 min, the tube was removed from the water bath and HBSS was added until a total volume of 50 ml was reached. The larger tubular fragments were allowed to settle under unit gravity for 10 min. The supernatant was removed and again fragments and cell clumps in this supernatant were allowed to settle for 10 min. From this tube, the supernatant was collected, filtered through a 60 m gauze and centrifuged for 2.5 min at 200 ***g***. After centrifugation, the supernatant was removed and the pellet was resuspended in 2 ml culture medium consisting of RPMI 1640 supplemented with penicillin (100 U/ml), streptomycin (100 g/ml), 0·1% bovine serum albumin (BSA) and glutamine (2 mM; all substances were obtained from Gibco). The supernatant collected after centrifugation was centrifuged again for 2.5 min at 200 ***g*** and this pellet was also resuspended in 2 ml culture medium. Both cell suspensions were put together. Cells were seeded either in 24-well plates on glass coverslips at a density of 150 000 cells

per well or in 96-well plates at a density of 75 000 cells per well. The cell isolates consisted of myoid cells, fibroblasts, macrophages, spindle-shaped mesenchymal-like cells, Sertoli cells, foetal-type Leydig cells and, depending on the age of the rat at the time of cell isolation, Leydig cell progenitors. The spindle-shaped mesenchymal-like cells, which in the testis can be found in a peritubular as well as a perivascular location (Haider 2004), are considered to be the stem cells/precursor cells of the Leydig cell progenitors (Lording & De Kretser 1972, Hardy *et al.* 1990, Teerds *et al.* 1999). The reason why we chose to use a relatively impure interstitial cell preparation was to include those cell types that have been shown *in vivo* to secrete paracrine factors known to influence the differentiation of stem Leydig cells, such as the Sertoli cell and peritubular/myoid cell-derived growth factors, PDGF-A, transforming growth factor-α (TGF-α)/epidermal growth factor (EGF), stem cell factor (SCF) and LIF (Ge *et al.* 2006).

The cells were cultured in RPMI 1640 medium with additions, as indicated above, for either 3 h, 3, 8 or 15 days at 37 °C in air plus 5% CO_2 in the presence of 0, 0·01, 0·1 or 10 ng/ml ovine LH respectively (NIH-LH-S20, Endocrinology Study Section of the National Institute of Health, Bethesda, MD, USA). In some experiments, oestradiol (Sigma) or DHT (Sigma) were also added to the culture medium at a concentration of 0·5 and 26 M respectively. The medium was replaced every 2 days except when the cells were cultured in the presence of DHT, in which case the medium was replaced daily.

The experiments described in the present study have been approved by the ethical committee for laboratory animal welfare of the Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands.

cAMP production

Hormone responsiveness of the isolated cells was measured by the production of cAMP in the culture medium. Interstitial cells isolated from 13-day-old rat testes were cultured in 24-well plates for 3, 8 or 15 days in the presence of 0, 0·1 or 10 ng/ml LH (NIH). At the end of the culture period, medium was removed and replaced by fresh medium. Cells were cultured for another 2 h in the presence of 0·1% BSA, 0·1 mM 3-isobutyl-1- methylxanthine (IBMX; Sigma) and 0 or 100 ng/ml LH (NIH) after which the medium was collected for measurement of cAMP production. Precursor cell development into progenitors was checked in these cultures using 3β-HSD enzyme histochemistry.

cAMP was assayed according to the method described by Harper & Brooker (1975) with minor modifications as reported by Schipper *et al.* (1996). The cAMP antibody used in the assay was a gift from Dr Stoof (Free University, Amsterdam, The Netherlands). The assay was validated for the use of culture medium and corrected for the presence of serum in the media samples. All samples were assayed in duplicate. The sensitivity of the assay was 0·125 pmol/ml and the inter- and intra-assay coefficients of variation were 2 and 8% respectively.

3β-HSD enzyme histochemistry and cell counts

Interstitial cells isolated from 10-, 13- or 18-day-old rats were cultured on glass coverslips in the presence of 0, 0·01, 0·1 or 10 ng/ml LH (NIH) in 24-well plates. After 3 h, 3 or 8 days of culture, 3β-HSD activity was determined in the cultures according to the method of Lojda *et al.* (1979) with minor modifications as described by Molenaar*et al.* (1986*a*). On each coverslip a total of at least 1000 cells was counted (3β-HSD-positive plus -negative cells), using a Nikon Optiphot 2 microscope, equipped with differential interference contrast optics. The number of blue-stained 3β-HSD-positive cells was expressed as percentage of the total number of cells counted per coverslip. In control experiments in which the enzyme substrate 5α-androstane-3β-ol-17-one was left out, no blue staining was observed.

Cell density assay

In order to investigate the viability of the cells in the cultures, methylene blue uptake by the cells was measured, using a colorimetric assay (Goldman & Bar-Shavit 1979). Briefly, interstitial cells isolated from 10-, 13- or 18-day-old rats were cultured in 96-well plates for 3 h, 3 or 8 days in the presence of 0, 0·01, 0·1 or 10 ng/ml LH (NIH) followed by fixation in 2·5% buffered formaldehyde. The cells were washed twice in 10 mM borate buffer (pH 8·5), stained with 1% methylene blue in borate buffer for 10 min at 24 °C and then rinsed with borate buffer. Specific cell-incorporated methylene blue was dissolved in 0·1 M HCl (60 min, 37 °C) and determined by reading the absorbance at 595 nm using a photospectrometer. The uptake of methylene blue was linearly correlated with the number of viable cells (Goldman & Bar-Shavit 1979).

Scintillation counting

The proliferative capacity of the interstitial cell preparations was determined by culturing cells isolated from 13-day-old rat testes in 96-well plates (75 000 cells/well) in the presence of 0 or 10 ng/ml LH for 1, 3 or 8 days. During the last 18 h of the culture period, [^{3}H]thymidine (2 μCi/ml; SA, 0·5 Ci/mmol; Amersham), a marker for cell proliferation which is incorporated in cells in the S-phase of the cell cycle, was added to the cultures. Cells were harvested onto glass fibre filters and the amount of incorporated radioactivity was determined using a liquid scintillation analyser (Beta plate, LKB, Turku, Finland).

In situ *hybridisation*

Interstitial cells isolated from 13-day-old rats were cultured on coverslips for 8 days in the presence of 0 or 10 ng/ml LH (NIH), fixed in buffered formalin for 15 min and washed with

PBS. The DIG RNA labelling kit (Boehringer, Mannheim, Germany) was used for the detection of 3β-HSD mRNA in these cells, according to the manufactures protocol with minor modifications. In brief, endogenous alkaline phosphatase activity was blocked by treatment with 0·2 M HCl and 0·3% Triton X-100 in PBS, followed by pre-incubation in proteinase K buffer for 30 min at 37 °C. Cells were then incubated in 10 g/ml proteinase K in the same buffer at 37 °C for 30 min. The reaction was stopped by transfer of the coverslips to a 0·2% ice-cold glycine solution for 1 min. Next, the coverslips were covered with 200 µl pre-hybridisation solution (5× SSC, 5× Denhardt's solution, 50% deionised formamide, 250 µg/ml yeast t-RNA, 250 µg denatured salmon sperm DNA, 4 mM EDTA) and incubated for at least 3 h at 50 °C. The coverslips were rinsed in alcohol, dried and covered with hybridisation solution, consisting of the pre-hybridisation solution (without salmon sperm DNA) to which 10 ng digoxygenin-labelled 3β-HSD cRNA probe (gift from Dr Mason, University of Edinburgh, Edinburgh, Scotland) per 20 µl of hybridisation solution was added. Hybridisation proceeded overnight at 52 °C in a humified chamber.

The hybridisation probe was detected by an alkaline phosphatase-coupled anti-digoxygenin antibody according to the manufacturer's instructions (Boehringer) with minor modifications. Briefly, the coverslips were rinsed in buffer A (0·1 M Tris–HCl pH 7·9, 0·15 M NaCl), blocked by incubation with 0·5% blocking reagent (Boehringer) in buffer A and rinsed again with buffer A. Next, the coverslips were incubated with an alkaline phosphatase conjugated anti-digoxygenin antibody (Boehringer) diluted 1:5000 in buffer A, rinsed twice in buffer A and equilibrated in buffer B (0·1 M Tris–HCl pH 9·5, 0·1 M NaCl, 0·05 M $MgCl_2$) before applying the colour detection substrate solution (175 g/ml 5-bromo-4-chloro-3-indolyl-phosphate (Boehringer) and 337·5 g/ml nitroblue tetrazolium (NBT; Boehringer)) to buffer B. The coverslips were incubated overnight in the dark and the reaction was stopped by rinsing the coverslips in Tris–HCl (0·1 M, pH 8·1) EDTA (1 mM) buffer after which the slips were mounted with fluoromount and stored at 4 °C in the darkness until further analysis.

Immunohistochemistry

LH receptor and 3β-HSD double labelling *in vitro*
Interstitial cells isolated from 13-day-old rats were cultured on glass coverslips in the presence of 0 or 10 ng/ml LH (NIH) for 1, 3 or 8 days, followed by fixation in 4% buffered formaldehyde. The cells were washed in 0·01 M PBS (pH 7·4), incubated with 0·1 M glycine in PBS for 30 min and rinsed again in PBS. Cells were permeablised by treatment with 0·1% Triton X-100 in PBS for 5 min after which the coverslips were rinsed thoroughly with PBS. The cells were blocked with 10% normal goat serum in PBS for 30 min and then incubated overnight at 4 °C with a combination of the two first antibodies: the polyclonal 3β-HSD antibody (a gift from Dr Payne, Stanford, CA, USA; diluted 1:100) and the monoclonal LH receptor antibody (P1B4, gift from Dr Wimalasema, University of Tennessee, Knoxville, TN, USA; diluted 1:2000) in 0·01 M PBS to which 0·05% BSA-c (Aurion, Wageningen, The Netherlands) was added. Cells were washed in PBS, followed by incubation for 1 h at room temperature with the second antibody mix: goat-anti-rabbit Alexa 568 (tetramethyl rhodamine isothiocyanate (TRITC), Molecular Probes Inc., Eugene, OR, USA) for the detection of 3β-HSD and goat-anti-mouse Alexa 488 (fluorescein isothiocyanate (FITC), Molecular Probes Inc.) for the detection of the LH receptor. Both antibodies were diluted 1:100 in 0·01 M PBS to which 0·05% BSA-c (Aurion) was added. To stop the incubation, the coverslips were rinsed several times in PBS, mounted with fluorsave and stored in the dark at 4 °C until microscopical examination. Fluorescent labelling for the presence of 3β-HSD and LH receptor was visualised using a Leica TCS MP confocal laser-scanning microscope (Leica, Heidelberg, Germany). The percentage of single- or double-labelled cells was determined by counting at least 200 fluorescent-labelled cells per culture. In control experiments, the first antibodies were replaced by normal rabbit serum (3β-HSD) and normal mouse serum (LH receptor). All controls showed a low level of irregular background labelling which could be easily distinguished from the specific labelling.

LH receptor and 3β-HSD double labelling *in vivo*
Testicular tissue from 7-, 8-, 10-, 12- and 13-day-old rats was collected, fixed in methacarn (60% methanol, 30% chloroform and 10% acetic acid) for 4 h at room temperature, dehydrated and embedded in paraffin. Five micrometre thick sections were cut and processed for immunohistochemistry as described previously (Teerds & Dorrington 1993, Teerds *et al.* 1999). Briefly, the sections were deparaffinised and endogenous peroxidase activity was blocked with 1% H_2O_2 in methanol. Slides were subsequently rinsed in PBS and incubated with 0·1 M glycine in PBS, blocked with 10% normal goat serum in PBS and incubated overnight at 4 °C with a combination of the two first antibodies 3β-HSD (diluted 1:300 in PBS/BSA-c) and LH receptor (diluted 1:1000 in PBS/BSA-c). Slides were rinsed and incubated with both secondary antibodies, a biotinylated horse-anti-mouse antibody and an alkaline phosphatase-labelled goat-anti-rabbit antibody (Vector Laboratories, diluted 1:200 in PBS/BSA-c) for 1 h. Sections were washed in PBS and incubated with the components avidin and biotin of the ABC staining kit elite (Vector Laboratories) for 1 h at a dilution of 1:1000 (in PBS/BSA-c). Slides were rinsed again and bound LH receptor antibody was visualised with 0·6 mg/ml 3,3′-diaminobenzidine tetrachloride (DAB, Sigma) dissolved in PBS to which 0·03% H_2O_2 was added (brown staining). Slides were rinsed in PBS and bound 3β-HSD antibody was visualised by incubating the slides with a solution containing 1 mg naphthol AS MX phosphate dissolved in 50 µl *N*,*N*-dimethylformamide, 8 ml Tris–HCl (0·1 M, pH 8·5), 2 mg

fast blue base dissolved in 50 µl of 2 N HCl and 50 µl 4% $NaNO_2$ (all chemicals obtained from Sigma) for ~15 min at 37 °C to demonstrate the presence of alkaline phosphatase (blue staining). The slides were rinsed and mounted; the sections were not counterstained. In control experiments, the primary antibodies were replaced by normal rabbit and mouse serum. Non-specific staining was not observed in these control sections.

BrdU and 3β-HSD double labelling *in vitro* Interstitial cells were isolated form 13-day-old rat testes, plated on glass coverslips and cultured for 1 or 3 days in the presence of 0 or 10 ng/ml LH (NIH). Three hours before termination of the experiment, bromodeoxyuridine (BrdU; 3·3 µg/ml, Sigma) was added to the cultures. Cells were fixed in methacarn for 10 min and then stored at 4 °C in 70% alcohol until further processing. BrdU is incorporated in the DNA of cells in the S-phase of the cell cycle and thus a maker for the proliferative activity of the cells. Immunohistochemical labelling was carried out as described before (Slegtenhorst-Eegdeman *et al.* 1998) with minor modifications. Briefly, the cells were pretreated with periodic acid prior to incubation with a monoclonal antibody (MAB) against BrdU (diluted 1:100, Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) for 2 h at room temperature, rinsed and incubated with a peroxidase-conjugated rabbit-anti-mouse antibody (diluted 1:100, Sigma). Labelling was visualised using DAB (Sigma) enhanced by nickel. After this incubation, the cells were extensively rinsed with PBS and incubated overnight at 4 °C with the polyclonal antibody against 3β-HSD as described above. Labelling was visualised using DAB (Sigma). In the control experiment, the 3β-HSD antibody was replaced by normal rabbit serum. Double-labelled cells were recognised by a dark brown to black nucleus (BrdU) and brownish stained cytoplasm (3β-HSD). Single-labelled cells had either a brown to black nucleus or brownish stained cytoplasm.

Statistical analysis

To test for significant differences in multi-comparisons, ANOVA was used, followed by Bonferroni's *post hoc* test if equal variances could be assumed or Dunnett's *post hoc* test to correct for unequal variances. Differences were considered to be significant when $P<0{\cdot}05$. In general, the experiments were performed in triplicate or quadruplicate and repeated at least thrice using different cell preparations. All data are expressed as mean ± S.D.

Results

Hormone responsiveness

cAMP levels in the culture medium were measured to investigate whether LH receptors in Leydig cell progenitors and presumably their precursors could be activated under the present experimental conditions. Interstitial cells were isolated from testes of 13-day-old rats and cultured for 3, 8 or 15 days in the presence of 0, 0·1 or 10 ng/ml LH. Under basal conditions, cells cultured without any additions or in the presence of 0·1 ng/ml LH produced comparable low amounts of cAMP, while cells that had been cultured in the presence of 10 ng/ml LH, produced two- to tenfold higher levels of cAMP when compared with control cells (Fig. 1A). When cells were stimulated with 100 ng/ml LH during the last 2 h of the incubation period, cAMP production was increased more than 100-fold (Fig. 1B) compared with under basal conditions (Fig. 1A). The capacity for maximal cAMP production was somewhat diminished in cells that had been cultured continuously in the presence of 10 ng/ml. From these results, it can be concluded that cultured Leydig cell progenitors and presumably their precursors remain

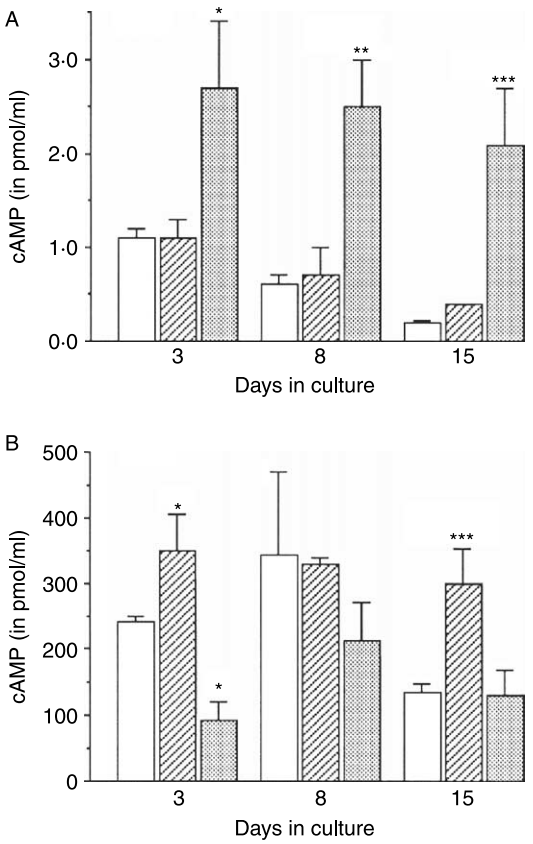

Figure 1 LH -induced cAMP production in interstitial cells. Cells were isolated from 13-day-old rat testes and cultured for 3, 8 or 15 days in the presence of 0 (open bars), 0·1 ng/ml (hatched bars) or 10 ng/ml (stippled bars) LH. At the end of the culture period, cells were incubated for 2 h either in fresh medium with 0·1 M IBMX (A) or in order to measure stimulated cAMP production in the presence of 100 ng/ml LH and 0·1 M IBMX (B). cAMP levels in the culture medium are expressed as pmol/ml culture medium. Values are presented as mean ± S.D. When no bar is present, the S.D. is too small to become visualized. *Significantly different from day 3 control cultures; **significantly different from day 8 control cultures; ***significantly different from day 15 control cultures, $P<0{\cdot}05$.

responsive towards LH in terms of cAMP production under the present experimental conditions.

3β-HSD enzyme histochemistry and cell counts

The percentage of 3β-HSD-positive cells was measured as an index of development of precursor cells into Leydig cell progenitors. The percentage of 3β-HSD-positive cells was < 2% in freshly isolated interstitial cell preparations from 10- to 13-day-old rats (Figs 2A, B and 3A, C) and 10–12% in cell preparations from 18-day-old animals (Figs 2C and 3E). The 3β-HSD-positive cells present 3 h after isolation in the cultures from 10-day-old testes are foetal-type Leydig cells; in the cultures with cells isolated from 13-day-old rats, the 3β-HSD-positive cells consisted of a mix of foetal-type Leydig cells and some newly developed Leydig cell progenitors though the number of progenitors will be limited. In the freshly isolated cell preparations from 18-day-old rat testes, the population of 3β-HSD-positive cells largely consisted of Leydig cell progenitors, since at this age, the progenitor Leydig cells will have outnumbered the foetal-type cells (Kerr & Knell 1988, Ariyaratne *et al.* 2000). We did not undertake any effort to discriminate foetal-type Leydig cells and progenitor cells from each other in the cultures due to the absence of appropriate markers that can discriminate 100% successfully between these two cell types.

Interstitial cells isolated from 10-day-old rats showed a twofold increase in the percentage of 3β-HSD-positive cells after being cultured in the presence of 0·1 or 10 ng/ml LH for 3 days (Fig. 2A). After 8 days of culture, the percentage of 3β-HSD-positive cells had increased fourfold. Although the percentage of 3β-HSD-positive cells did not increase significantly when cells were cultured without LH, some autonomous progenitor formation did seem to take place (Figs 2A and 3B). In a few cases, interstitial cells were isolated from testes of 8-day-old rats. In these experiments, the percentage of 3β-HSD-positive cells that had developed after 8 days of culture in the presence of LH was slightly lower, though not significantly different from the percentage of progenitor cells formed when cells were isolated from 10-day-old rat testes (data not shown). When interstitial cells were isolated from 13-day-old rats, a fourfold increase in the percentage of 3β-HSD-positive progenitor cells was observed after 3 days of culture without LH, suggesting that these cells underwent spontaneous autonomous differentiation (Fig. 2B). Addition of LH further augmented this differentiation process in a dose-dependent manner (Fig. 2B). After 8 days of culture in the presence of LH, the percentage of 3β-HSD-positive cells had even further increased. In the absence of LH, however, no further increase in the percentage of 3β-HSD-positive Leydig cell progenitors was observed after prolonged culture (Figs 2B and 3D). Interstitial cells isolated from 18-day-old rats did not show any spontaneous differentiation into 3β-HSD-positive Leydig cell progenitors. High doses of LH and prolonged culture periods were required for the stimulation of precursor cell differentiation

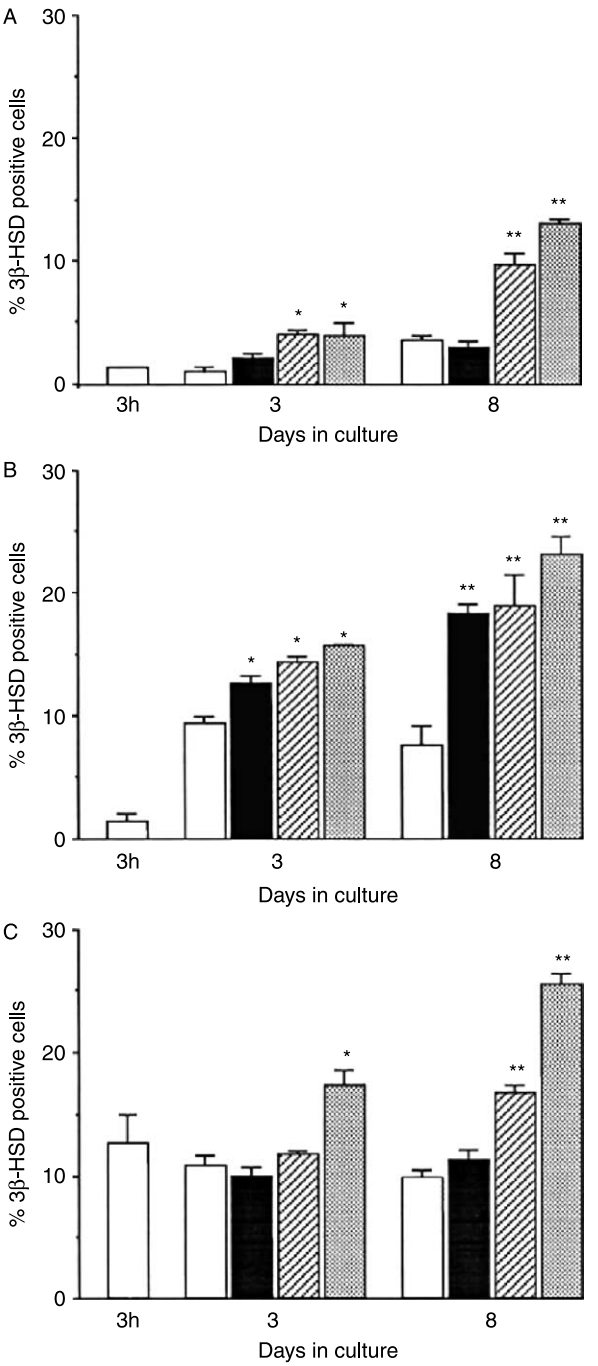

Figure 2 Differentiation of mesenchymal-like precursor cells into 3β-HSD-positive Leydig cell progenitors. Interstitial cells were isolated from (A) 10-, (B) 13- and (C) 18-day-old rat testes and cultured in the presence of 0 (open bars), 0·01 ng/ml (filled bars), 0·1 ng/ml (hatched bars) or 10 ng/ml (stippled bars) LH during 3 h, 3 or 8 days. At the end of the culture period, the percentage of 3β-HSD-positive cells was determined. The results represent the means of quadruplicate cultures from one representative experiment; values are expressed as mean ± S.D. When no bar is present, the S.D. is too small to become visualized. *Significantly different from day 3 control cultures; **significantly different from day 8 control cultures, $P<0·05$.

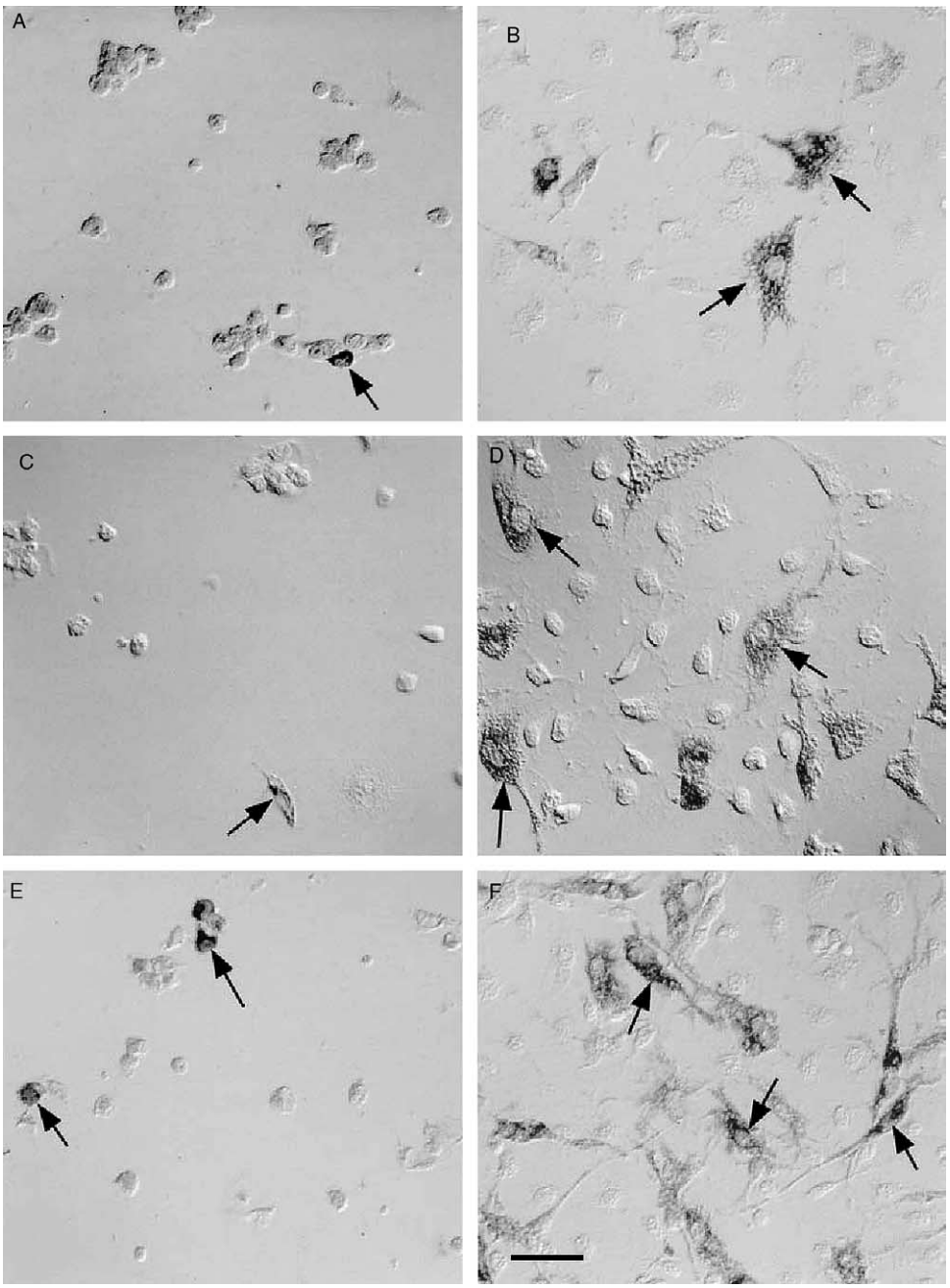

Figure 3 3β-HSD enzyme histochemistry: representative examples of cell cultures. Interstitial cells were isolated from (A and B) 10-, (C and D) 13- or (E and F) 18-day-old rat testes. Cells after 3 h of culture (A, C and E) in the absence of LH, or 8 days of culture (B, D and F) in the presence of 10 ng/ml LH, 3β-HSD-positive cells stained blue; some representative cells are indicated by arrows. Original magnification 574×, bar 17 m.

into 3β-HSD-positive cells (Figs 2C and 3F). It cannot be excluded that in this case after 8 days of culture in the presence of LH some progenitor cells may have already differentiated into immature Leydig cells.

Independent of the age of the animals at the time of interstitial cell isolation, the culture conditions of the cells or the length of the culture period, no significant changes in cell numbers between controls and LH-treated cells could be detected using the methylene blue cell viability assay (data not shown).

In order to investigate whether proliferation of cells in culture took place, [^{3}H]thymidine or BrdU, both markers for

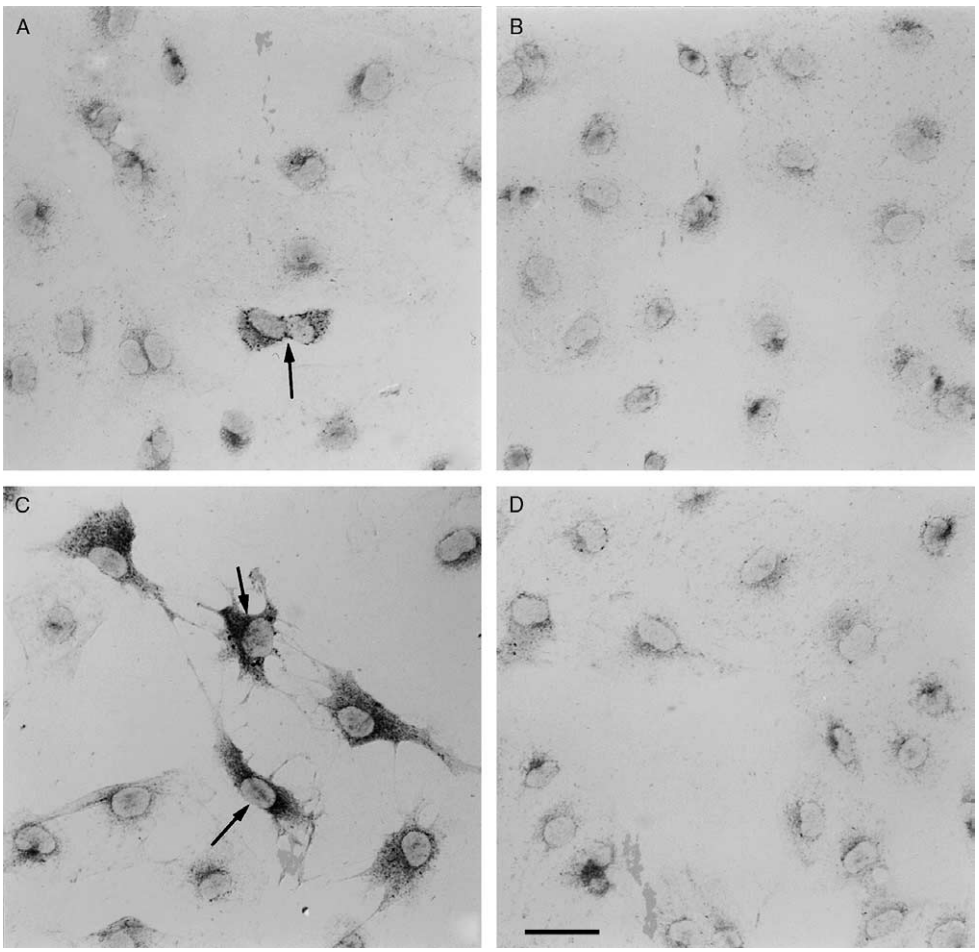

Figure 4 *In situ* hybridisation of 3β-HSD mRNA. Interstitial cells were isolated from 13-day-old rat testes and cultured for 8 days in the absence (A and B) or the presence (C and D) of 10 ng/ml LH (A, C anti-sense probe and B, D sense probe). Cells expressing 3β-HSD mRNA stained blue, some cells are indicated by arrows. Original magnification 574×, bar 17 m.

cell proliferation, were added to interstitial cells that were isolated from 13-day-old rat testes and cultured without any additions or in the presence of 10 ng/ml LH for either 1, 3 or 8 days. Neither after 1 day of culture nor after 3 and 8 days of culture a significant difference in the incorporation of [^{3}H]thymidine was observed (day 1: control 69±22 CCPM/75,000 cells, LH 86±23; day 3: control 42±23, LH 54±11; day 8: control 41±9, LH 62±15).

In another experiment, cells were double-labelled with antibodies against BrdU and 3β-HSD to identify Leydig cell progenitors. Neither after 1 day of culture nor after 3 days of culture, the percentage of double-labelled Leydig cell progenitors differed significantly from zero, nor did the percentage of double-labelled cells differ significantly between the control and the LH-treated cultures (day 1: control 0·1±0·1% of cells was double-labelled, LH 0·2± 0·2%; day 3: control 0·1±0·1%, LH 0·3±0·1%). The percentage of single-labelled BrdU-positive cells also did not differ significantly between the control and LH-treated cells after 1 and 3 days of culture (day 1: control 3·7±1·4% of cells labelled, LH 3·1±1·3%; day 3: control 0·2±0·2%, LH 0·4±0·1%). The BrdU labelling experiment was not expanded up to 8 days of culture because by that time the cells had ceased to proliferate (Teerds, unpublished data). Since these observations fitted well with the results of the methylene blue cell viability assay (see above), the [^{3}H]thymidine incorporation and double-labelling experiments were not continued in the other treatment groups.

Under none of the culture conditions as described in this study, an effect on the percentage of 3β-HSD-positive cells was observed following daily addition of 26 M DHT either alone or in combination with LH (data not shown). Precursor cell differentiation was not affected by the addition of oestradiol. Only a dose of 0·5 μM oestradiol caused a slight but significant (30%) inhibition in LH-stimulated progenitor cell formation when cells were isolated from 13-day-old rats

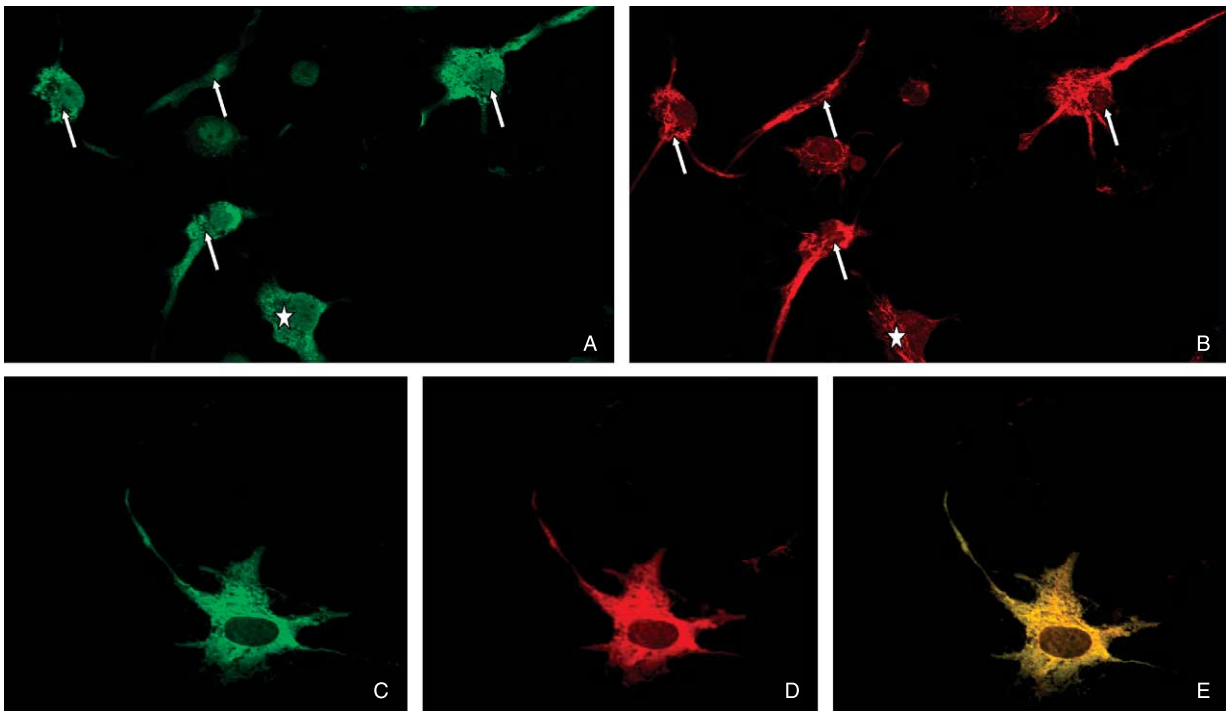

Figure 5 Immunohistochemical double staining for the concomitant detection of LH receptors and 3β-HSD. Interstitial cells were isolated from 13-day-old rat testes and cultured for 1 day without addition of LH. Following fixation, the cells were double stained with antibodies against the LH receptor and 3β-HSD. Cells were studied using a Leica confocal laser-scanning microscope. (A and C) Green LH receptor-positive cell(s) visualized using the FITC channel; (B and D) same cell(s) stained for the presence of 3β-HSD, visualized using the TRITC channel; (E) same cell(s) from C and D now visualized through both the FITC and TRITC channel (yellow fluorescence). In A and B, double-labelled cells are indicated by arrows, a cell which stained positive only with the LH receptor antibody and in which the 3β-HSD staining did not reach above the background level is indicated by an asterisk. Original magnification 1732×.

and cultured for 8 days in the presence of 10 ng/ml LH ($P<0{\cdot}05$).

3β-HSD in situ *hybridisation*

The detection of LH-induced changes in 3β-HSD enzyme activity as an index of cell differentiation was validated by comparing the effects of LH on the number of cells expressing 3β-HSD mRNA. Interstitial cells were isolated from testes of 13-day-old rats and cultured without LH or in the presence of 10 ng/ml LH. After a culture period of 8 days, the increase in the percentage of 3β-HSD mRNA expressing Leydig cell progenitors was similar to that observed after histochemical detection of 3β-HSD enzyme activity (Fig. 4). Therefore, the *in situ* hybridisation experiments for 3β-HSD mRNA expression were not continued for interstitial cells isolated from either 10- or 18-day-old rats, or with other doses of LH.

3β-HSD and LH receptor immunohistochemistry

The possible co-localisation of LH receptors and 3β-HSD in mesenchymal-like precursor cells and Leydig cell progenitors was investigated by immunohistochemistry (Fig. 5). Interstitial cells were isolated from 13-day-old rats and cultured without or in the presence of 10 ng/ml LH for 1, 3 or 8 days. Immunohistochemical detection of these two antigens was determined by fluorescent confocal laser scanning microscopy: the LH receptor was detected using the FITC (green) channel and 3β-HSD was detected by using the TRITC (red) channel. In each culture, a minimum of 200 single- and/or double-labelled cells was counted. After 1 day of culture without LH $9{\cdot}5\pm3{\cdot}5\%$ of the fluorescent cells counted stained positively with only the LH receptor antibody, while the percentage of double-labelled cells (3β-HSD and LH receptor positive) was $90{\cdot}5\pm3{\cdot}5$ (Fig. 5A–C). Cells staining positively for 3β-HSD alone were not detected. After culturing the cells for 3 days in the presence of LH, the percentage of single-labelled LH receptor-positive cells had become significantly lower ($2{\cdot}8\pm1{\cdot}6$; $P<0{\cdot}05$) compared with the controls. Concomitantly, the percentage of double-labelled cells had increased up to $97{\cdot}2\pm1{\cdot}6$ in these cultures. After 8 days of culture in both the control and the cultures treated with LH, the percentage of cells that stained positively for the LH receptor alone had become negligible.

In an additional *in vivo* experiment, paraffin-embedded sections of testes from 7-, 8-, 10-, 12- or 13-day-old rats were double-labelled with the antibodies against the LH receptor

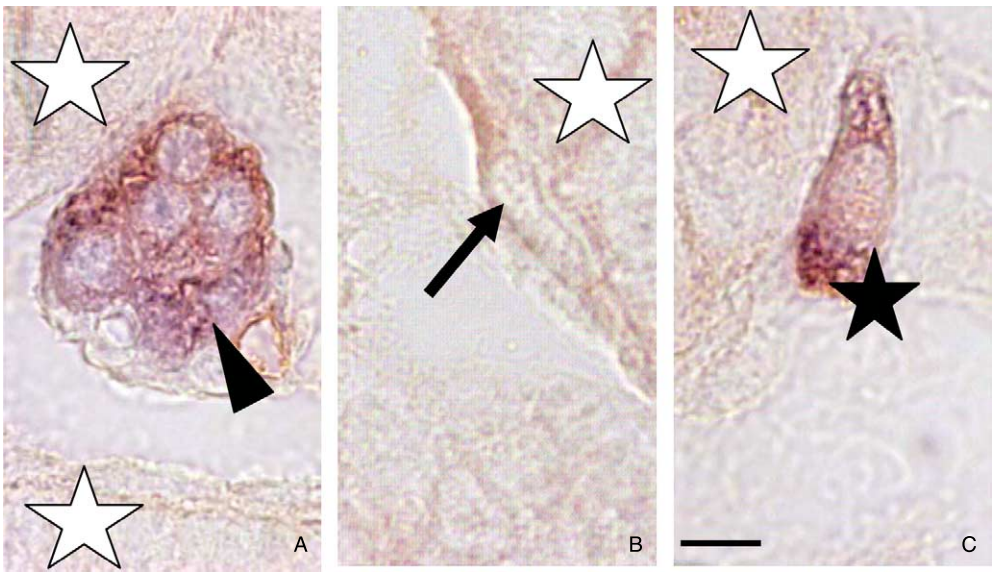

Figure 6 Immunohistochemical double staining for the concomitant detection of LH receptors and 3β-HSD in the prepubertal testis. (A) Cluster of double stained (LH receptor (brown) and 3β-HSD (blue))-positive foetal-type Leydig cells (arrowhead) in the testis of a 7-day-old rat. A seminiferous tubule is indicated by a white asterisk; (B) peritubularly located spindle-shaped precursor cell (black arrow) in the testis of a 12-day-old rat staining positively (brown) with the LH receptor antibody. No 3β-HSD staining (blue) could be detected in this cell; (C) peritubularly located double-labelled (LH receptor and 3β-HSD positive) oval-shaped Leydig cell progenitor in the testis of a 13-day-old rat. The sections were not counterstained. Original magnification 1435×, bar=6·8 μm.

and 3β-HSD. In 7-, 8- and 10-day-old testes foetal-type Leydig cells were the only cell type that stained positively with both antibodies (Fig. 6A). In the testes of 12-day-old rats, spindle-shaped cells were observed that stained positively with the LH receptor antibody, while no 3β-HSD immunoreactivity could be detected in these cells (Fig. 6B). In testes of 13-day-old rats, the first double-labelled spindle- to oval-shaped cells were observed (Fig. 6C). These *in vivo* data confirm our *in vitro* observations that precursor cells acquire LH receptors before they differentiate into progenitor cells.

Discussion

In the present study, the role of LH in the formation of LH receptor/3β-HSD-positive Leydig cell progenitors was investigated *in vitro*. In order to obtain stem Leydig cells/precursor cells at different stages of the developmental process, interstitial cells were isolated from rats at the ages of 10, 13 and 18 days. The presence of 3β-HSD enzyme activity and/or the presence of LH receptors were used as differentiation parameters. Cells that expressed only LH receptors were considered to be precursor cells that had developed from stem Leydig cells (Ge *et al.* 2006), while cells that expressed both LH receptor and 3β-HSD protein were considered to be Leydig cell progenitors. Independent of the age at the time of cell isolation or the duration of the culture period, no cells were observed that stained only positive with the 3β-HSD antibody. This was confirmed by an *in vivo* double-labelling experiment in which in 12-day-old rat testes, single-labelled LH receptor-positive cells could be observed, but no single-labelled 3β-HSD-positive cells were found. When *in vitro* a dose of LH as low as 0·1 ng/ml was added to the cultures, the percentage of 3β-HSD-positive Leydig cell progenitors increased in nearly all cases significantly. Independent of the age of the animals at the time of interstitial cell isolation, no appreciable cell proliferation could be detected at any time during culturing, indicating that the increase in the percentage of 3β-HSD-positive cells *in vitro* was most likely the reflection of differentiation of stem Leydig cells/precursor cells rather than division of foetal-type Leydig cells or newly formed Leydig cell progenitors.

The responsiveness of the LH receptor system was intact in terms of stimulation of cAMP production, even after 15 days of culture. The maximal capacity for the production of cAMP in the presence of 100 ng/ml LH was lower in cells that were cultured in the continuous presence of 10 ng/ml LH. This is very likely a reflection of desensitisation of the LH receptor system (Hsueh *et al.* 1977, Nozu *et al.* 1981, Calvo *et al.* 1984, LaPolt *et al.* 1991, Amsterdam *et al.* 2002).

Our hypothesis that LH is essential for the differentiation of precursor cells into Leydig cell progenitors is based on observations in adult rats. After the destruction of the existing Leydig cell population by the cytotoxic drug ethane dimethyl sulphonate (EDS), regeneration of a new adult-type Leydig

cell population occurred only in the presence of LH (Molenaar *et al.* 1986*b*, Veldhuizen-Tsoerkan *et al.* 1994, Teerds 1996, Tena-Sempere *et al.* 1997, Teerds *et al.* 1999). This regeneration process shows many similarities with the development of the adult-type Leydig cell population in the (pre)pubertal testis (Teerds 1996). Recently, however, some groups have questioned the role of LH in the initiation of progenitor Leydig cell development. In LH receptor knockout (LuRKO) mice testes, Zhang *et al.* (2001, 2002) showed by immunohistochemistry that in the absence of LH receptor signalling at days 10 and 20 after birth a few $P450_{17\alpha}$ and 3β-HSD type I positively labelled cells could be detected in close vicinity of the seminiferous tubules. The morphology of these cells resembles the morphology of Leydig cell progenitors as does the location where the labelled cells are found, suggesting that a limited number of progenitors may have develop in the absence of active LH receptor signalling. These authors concluded that LH receptor signalling is important for Leydig cell differentiation after birth, though the initiation of adult Leydig cell differentiation in the mouse testis can begin independently of LH action. Our present *in vitro* study expands these observations by showing that it seems to be only a small subpopulation of cells that can initiate differentiation independent of LH signalling. For the majority of the stem Leydig cells/precursor cells, the presence of LH is essential to induce normal differentiation into 3β-HSD-positive progenitor cells.

The levels of some steroidogenic enzymes were severely reduced in the adult LuRKO mice testes, while the expression of thrombospondin 2, a marker for foetal-type Leydig cells, was not reduced (Zhang *et al.* 2001, 2004). The levels 3β-HSD type VI mRNA expression, a marker for adult-type Leydig cells, were only slightly higher in testes of 60-day-old LuRKO mice compared with 10- and 20-day-old LuRKO and wild-type mice. These observations made the authors conclude that the majority of Leydig cells present in the adult LuRKO mouse testis will be of foetal origin (Zhang *et al.* 2004). It is not clear from these studies whether the progenitor cells that have developed in the prepubertal LuRKO testis persist during adulthood. Clearly, the nature of the Leydig cells in the adult LuRKO testis needs further investigation.

In another study, Baker *et al.* (2003) reported that in the testes of adult hpg mice genes were expressed, which are present only in the adult-type Leydig cell lineage, such as 3β-HSD type VI and 17β-HSD type III. The number of Leydig cells present in adult hpg mice was, however, only 10% of wild-type mice (Baker & O'Shaughnessy (2001). Although we also found indications that differentiation of some precursor cells into Leydig cell progenitors may have occurred independently from LH, their number was limited. It is, however, questionable whether hpg mice which have a defect in the synthesis of gonadotrophin releasing hormone (GnRH) but which are not a GnRH knockout, completely lack circulating LH. It may depend on the sensitivity of the LH RIA that has been used whether investigators have been able to detect of LH (and follicle-stimulating hormone, FSH) in plasma of hpg mice (van Casteren *et al.* 2000). Some investigators have reported that LH levels in hpg mice were undetectable (e.g. Baker *et al.* 2003), while others presumably using a more sensitive RIA showed that LH levels in these mice varied between $<0{\cdot}2$ and 0·5 ng/ml (Gibson *et al.* 1994, Barkan *et al.* 2005). The results of the present *in vitro* study demonstrated that adult-type Leydig cell development can take place in the presence of an amount of LH as low as 0·01 ng/ml, levels that cannot be measured by RIA at present (van Casteren *et al.* 2000). *In vitro* conditions are of course not similar to the *in vivo* situation and, therefore, one has to be cautious when extrapolating *in vitro* data to the *in vivo* situation. Nevertheless, the observations of the present study do suggest that the very low levels of circulating LH in hpg mice may possibly be high enough to induce the formation of mature adult-type Leydig cells. This could also explain why Baker *et al.* (2003) were able to detect specific markers for mature adult-type Leydig cells, such as 17β-HSD, in the testes of adult hpg mice, while Zhang and colleagues were unable to detect this enzyme in the adult LuRKO testis. Whether the low levels of LH in hpg mice are indeed of influence on progenitor formation needs to be further investigated by for instance immunising hpg mice from birth to adulthood against LH, thus eliminating the effect of residual LH on this developmental process. It would be of interest to determine what happens to the final number of Leydig cells in the adult hpg testis under these conditions and whether these cells express more adult-type steroidogenic enzymes or more enzymes characteristic for less well-differentiated Leydig cells such as progenitor cells.

Our hypothesis that LH is essential for the normal development of Leydig cell progenitors is further confirmed by the immunohistochemical LH receptor/3β-HSD double-labelling experiments. We never observed a cell that was solely labelled with the 3β-HSD antibody, neither *in vitro* nor *in vivo*. On the other hand, cells that stained positively with only the LH receptor antibody were detected *in vitro* regularly. *In vivo,* these cells were most often observed in a peritubular location in testes of 12-day-old rats, while spindle- to oval-shaped LH receptor and 3β-HSD-positive progenitor cells were observed for the fist time in testes of rats at 13 days post partum (pp). These results are in contrast to what has been reported by Ariyaratne *et al.* (2000), who showed by immunohistochemistry that *in vivo* Leydig cell progenitors first express 3β-HSD as well as other steroidogenic enzymes before the LH receptor becomes detectable. These authors concluded that the development of spindle-shaped precursor cells into progenitor cells took place independently from LH. The discrepancy with the *in vitro* and *in vivo* data in the present study cannot be explained by the use of different LH receptor antibodies, since Ariyaratne *et al.* (2000) employed the same LH receptor antibody as used in the current experiments. A possible explanation for this contradiction may be that Ariyaratne and colleagues did not use immunohistochemical double labelling to determine the presence of LH receptors and 3β-HSD in the same spindle-shaped cell, but used serial

sections. Especially, the spindle-shaped precursor cells are relatively small and therefore the amount of cytoplasm present in the sections may have been too limited to be able to detect the LH receptor and 3β-HSD antigen in the same cell in serial sections. Furthermore, the techniques used in the present study to detect the two antigens, immunofluorescence and three-step immunohistochemistry (using the Vector Stain kit elite) were more sensitive than the detection method used by Ariyaratna and colleagues.

In order to exclude that the increase in the percentage of Leydig cell progenitors was the result of cell proliferation and not differentiation of precursor cells, we determined the viability of the cells using the methylene blue cell viability assay as well as the incorporation of the proliferation markers [^{3}H]thymidine and BrdU. No significant differences in cell viability were observed at any time between the control and the LH-treated cultures. The percentage of 3β-HSD–BrdU double-labelled Leydig cell progenitors was negligible under all conditions, suggesting that the increases in the percentages of 3β-HSD-positive cells was the consequence of differentiation of precursor cells into Leydig cell progenitors and not the result of proliferation of foetal-type Leydig cells and/or newly formed Leydig cell progenitors.

Although the percentage of foetal-type Leydig cells in the cultures was low and these cells did not contribute to the increase in 3β-HSD-positive cells after 3 and 8 days of culture as discussed above, it would be appropriate if one could discriminate even after 8 days of culture between foetal-type Leydig cells and newly formed progenitor Leydig cells. As shown in the study by Zhang *et al.* (2004), there are at present no specific markers available that can discriminate 100% successfully between these two cell types. This group showed that adult-Leydig cell markers, such as 17β-HSD type III are not only expressed by adult Leydig cells and their progenitors, but also by foetal-type Leydig cells. The group of Mendis-Handagama has suggested that 11β-HSD type I may be a marker for progenitor Leydig cells that have developed between days 10 and 13 *post partum* since this enzyme was detected by immunohistochemistry in these cells, while at the same time, it was not present in foetal-type Leydig cells (Ariyaratne *et al.* 2000). This study, however, contradicts observations by other groups. Phillips *et al.* (1989) showed for instance that 11β-HSD immunoreactivity could not be detected before the age of 26 days in the rat testis when progenitors have nearly completed their differentiation into immature Leydig cells. The group of Hardy showed that 11β-HSD type I activity, mRNA and protein levels were very low to nearly absent in highly purified 21-day-old progenitor cells (Ge *et al.* 1997), confirming the observations by Philips and colleagues.

In several older studies, it has been suggested that foetal-type Leydig cells disappear from the testis relatively rapid after birth. Isolation of foetal Leydig cells among other interstitial cells from testes of 10- or 13-day-old rats could possibly 'rescue' these cells from undergoing degeneration, and these 'rescued' foetal-type Leydig cells could then contribute to the increasing number of 3β-HSD-positive cells during culture. We do not think, however, that this likely to occur, since Kerr & Knell (1988) have shown in an extensive morphometric analysis that the number of foetal-type Leydig cells after birth remains constant up to 2 weeks *post partum,* followed by a gradual decrease. Between the age of 21 and 100 days, the size of the foetal-type Leydig cell population remains relatively constant at 50–75% of its original size at the time of birth.

As indicated above, the results of the present study emphasis the importance of LH in the differentiation of precursor cells into Leydig cell progenitors. Nevertheless, under the present culture conditions differentiation of a subpopulation of precursor cells can apparently take place without the immediate presence of LH. Based on the present *in vitro* data, it is not possible to speculate about the exact size of this subpopulation of cells. Indications that heterogeneity may exist in the Leydig stem cell/precursor cell population also comes from older studies in which adult rats received daily injections of hCG. Within 2 days after the start of treatment the Leydig cell population had increased by 35% as a result of stem Leydig cell/precursor cell differentiation, Leydig cell proliferation being negligible. These newly formed Leydig cells have characteristics in common with progenitor cells (Teerds *et al.* 1988, 1992, Teerds 1996). Despite the observed heterogeneity in the adult testis, there are several possible explanations for the autonomous differentiation of this subpopulation of precursor cells into Leydig cell progenitors in the prepubertal testis. First, of all, these cells may have been primed by LH before isolation and no longer depend on the presence of LH in the culture medium. Secondly, the LH receptor on these precursor cells may show intrinsic activation during the first days of culture, making the immediate presence of LH less relevant for the differentiation process (personal communication of Prof. I Huhtaniemi).

When interstitial cells were isolated at the age of 10 days, it took between 3 and 8 days for LH to induce the formation of 3β-HSD-positive Leydig cell progenitors. This is probably a reflection of the fact that the stem Leydig cells/precursor cells at day 10 *post partum* are less well developed and need more time to differentiate into progenitor cells. This assumption is supported by the observation that a small though not significant increase in the percentage of 3β-HSD-positive cells in untreated control cultures was observed after 8 days of culture.

After isolation of interstitial cells at day 18 after birth only a marginal increase in the percentage of 3β-HSD-positive cells was observed after 3 days of culture in the presence of the highest dose of LH. Hardy *et al.* (1990, 1991) did not observe this small increase under their culture conditions, but these investigators used a dose of LH comparable with 0·1 ng/ml. We observed that in the presence of such a low dose of LH, it took between 3 and 8 days of culture to induce an increase in the percentage of 3β-HSD-positive cells. Another difference between the experimental design of the study by Hardy and the present one is that we isolated interstitial cells from 18-day-old animals, while these authors used 21-day-old rats.

We preferred to use 18-day-old animals since *in vivo* studies have demonstrated that at this age the wave of Leydig cell progenitor proliferation has not yet been initiated; this starts around the age of 21 days in rats (Hardy *et al.* 1989).

The present study also investigated the role of steroids in the regulation of Leydig cell development. In contrast to the group of Hardy (Hardy *et al.* 1990), we were unable to demonstrate a stimulating effect of daily treatment with large amounts of DHT (26 μM) on precursor cell differentiation *in vitro.* We did notice, however, that with increasing age at the time of cell isolation, higher doses of LH and longer culture periods were necessary to induce progenitor formation *in vitro.* Possibly, the addition of high doses of DHT and thus saturation of the androgen receptor has sensitised precursor cells isolated from 21-day-old rats for LH to enhance the efficiency of progenitor development, but not in cells isolated from 18-day-old animals. Oestradiol induced a small inhibitory effect on progenitor formation after 8 days of culture. The dose of oestradiol needed was, however, very high in relation to the concentrations present in body fluids under physiological conditions. Hence, non-specific effects of the added oestradiol cannot be excluded. Inhibitory effects of large doses of oestradiol on the development of adult-type Leydig cells have also been observed *in vivo* in prepubertal rats (Abney 1999) and in adult rats following administration of EDS (Abney & Myers 1991). Again, it cannot be excluded that this inhibition is caused by non-specific effects of the very high doses of oestradiol used in these studies.

Taken together, the regulation of the development of the adult-type Leydig cell population, as discussed above, is a complicated process. A summary is given in Fig. 7. In brief, spindle-shaped stem Leydig cells undergo proliferation and differentiation under the influence of the growth factors like LIF, SCF, PDFG-A and EGF/TGF-α, and the thyroid hormone triiodothyronine (T_3). This initial phase of differentiation does take place independent of LH (Ge *et al.* 2006). In the strain of rats used in the present study, the stem Leydig cells differentiate between days 10 and 12 after birth into spindle-shaped precursor cells which express LH receptors. Under the influence of LH, DHT (Hardy *et al.* 1990) and T_3, these cells develop, around the age of 13 days, into 3β-HSD-positive progenitor cells that have an elongated to oval shape and contain lipid droplets in their cytoplasm (Haider 2004, Ge *et al.* 2006). In the absence of LH or LH receptor signalling, formation of progenitor cells is severely inhibited, although a small subpopulation of cells can differentiate into progenitor cells without the immediate presence of LH (Zhang *et al.* 2004, the present study). Besides LH, the expression of Dhh (Clark *et al.* 2000) and the presence of macrophages (Gaytan *et al.* 1994) in the testis also appears to be important for this phase of the developmental process. Increased oestradiol levels inhibit the formation of progenitors at least in part (Abney 1999). The newly formed Leydig cell progenitors undergo several waves of proliferation (Hardy *et al.* 1989) under the influence of LH and local produced growth factors (for review see Haider 2004). In contrast to mature Leydig cells, the major steroids produced by progenitor cells are 5α-reduced androgens such as DHT (O'Shaughnessy *et al.* 2002, Haider 2004, Ge *et al.* 2005). Between days 28 and 35 after birth, the progenitors differentiate into immature polygonal-shaped Leydig cells that have still some lipid droplets in their cytoplasm; when these cells undergo further differentiation proliferation ceases and 5α-reductase levels decrease. At the same time, the levels

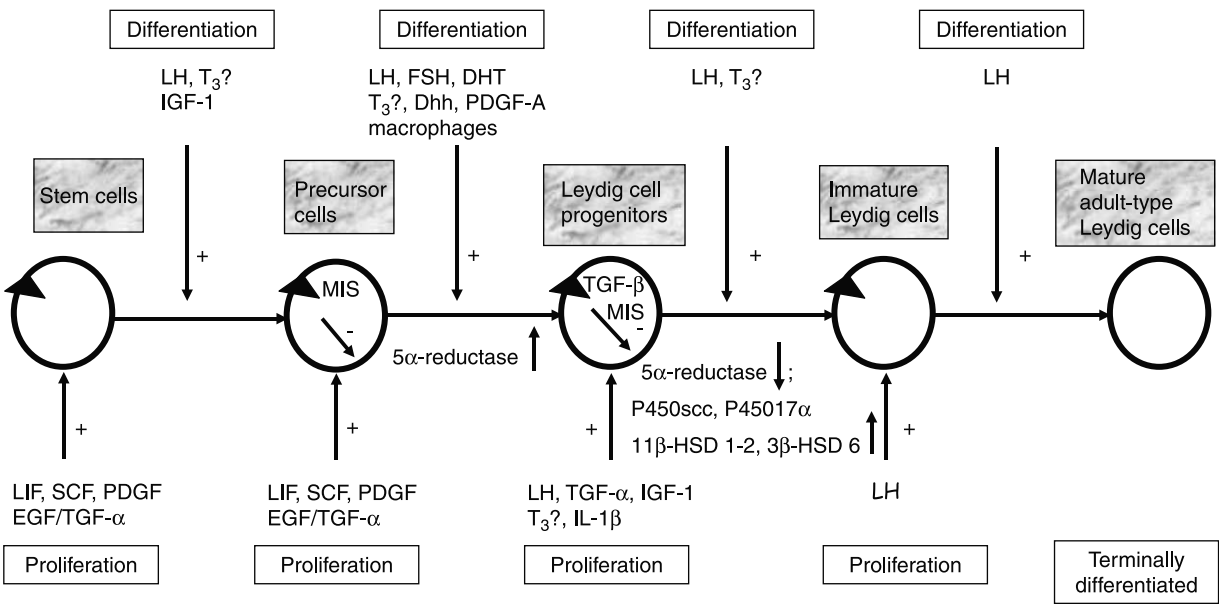

Figure 7 Integrated model for the development of adult-type Leydig cells in the (pre)pubertal testis. The model is explained in the Discussion section.

of other steroidogenic enzymes, such as $P450_{scc}$, $P450_{17\alpha}$, 11β-HSD type 1 and 2, as well as 3β-HSD type VI begin to increase (Wiebe 1976, Ge & Hardy 1998, O'Shaughnessy *et al*. 2000, 2002, Zhang *et al*. 2004, Ge *et al*. 2005). Around the age of 60 days, most immature Leydig cells have under the influence of LH and possibly other (growth) factors developed into terminally differentiated mature adult-type Leydig cells with a characteristic large round shape (Haider 2004). In conclusion, even though certain phases of the developmental process may take place in the absence of LH or LH signalling, it is clear that for the development of a normal sized adult-type Leydig cell population, LH is indispensable.

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