Leydig cell re-generation and expression of cell signaling molecules in the germ cell-free testis

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Abstract

Leydig cells in the rat testis can be specifically ablated with ethane dimethane sulfonate (EDS) and will subsequently re-generate. In this study, we have characterized Leydig cell re-generation and expression of selected cell-signaling molecules in a germ cell-free model of EDS action. This model offers the advantage that re-generation occurs on a stable background without confounding changes from the regressing and repopulating germ cell population. Adult rats were treated with busulfan to remove the germ cell population and Leydig cells were then ablated with EDS. Testicular testosterone levels declined markedly within 24 h of EDS treatment and started to recover after 8 days. After EDS treatment there were marked declines in levels of Leydig cell-specific mRNA transcripts coding for steroidogenic enzymes cytochrome P450 11a1 (*Cyp11a1*), cytochrome P450 17a1 (*Cyp17a1*), 3 β -hydroxysteroid dehydrogenase type 1 (*Hsd3b1*), 17 β -hydroxysteroid dehydrogenase type 3 (*Hsd17b3*) and the LH receptor. Levels of all transcripts recovered within 20 days of EDS treatment apart from *Hsd17b3*, which remained undetectable up to 20 days. Immunohistochemical localization of CYP11A1 during the phase of early Leydig cell re-generation showed that the Leydig cell precursors are spindle-shaped peritubular cells. Studies on factors which may be involved in Leydig cell re-generation showed there were significant but transient increases in platelet-derived growth factor A (*Pdgfa*), leukemia inhibitory factor (*Lif*), and neurofilament heavy polypeptide (*Nefh*) after EDS, while desert hedgehog (*Dhh*) levels declined sharply but recovered by 3 days. This study shows that the Leydig cell precursors are peritubular cells and that expression of *Pdgfa* and *Lif* is increased at the start of the re-generation process when precursor proliferation is likely to be taking place.

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Introduction

During normal male fetal development the Leydig cells actively secrete androgen to ensure normal masculinization. Androgen secretion then declines before a second generation of Leydig cells establishes the normal adult pattern of secretion during puberty (Klonisch *et al.* 2004). In the mouse and rat, the two Leydig cell populations appear to represent distinct cell lineages and show clear differences in their regulation and pattern of development. The fetal Leydig cells arise soon after testis differentiation around 14.5 days of intrauterine development (Majdic *et al.* 1998) while the adult population starts to develop just before puberty, around postnatal day 12 in the rat (Ariyaratne *et al.* 2000a). In the mouse, a number of factors have been identified which are involved in fetal Leydig cell differentiation. These include desert hedgehog (DHH), platelet-derived growth factor A (PDGFA), and the Arx homeobox (ARX) and, in each case, gene knockout studies have shown that these factors are required for normal fetal Leydig cell development (Kitamura *et al.* 2002, Yao *et al.* 2002, Brennan *et al.* 2003). In contrast, the factors

initiating and regulating development of the adult population of cells remain unclear. A recent study has reported the isolation of stem cells from the pre-pubertal testis, which give rise to Leydig cells *in vivo* following transplantation (Ge *et al.* 2006). These stem cells express receptors for PDGFA, leukemia inhibitory factor (LIF), and kit ligand (KITL) and proliferate in the presence of these mitogens (Ge *et al.* 2006). This would appear to suggest a central role for these growth factors, and in particular PDGF, in the development of the adult Leydig cell cohort.

Despite isolation of the Leydig cell stem cells, the identity of the precursor cells that give rise to the adult Leydig cells remains uncertain and controversial. A number of studies have indicated that Leydig cell precursor cells are predominantly peritubular, based on morphological and immunohistochemical evidence (Haider *et al.* 1995, Russell *et al.* 1995, Haider & Sevros 1998, Ariyaratne *et al.* 2000a, 2000b). A recent study, however, has reported that the progenitor cells are vascular smooth muscle cells and pericytes, based on the apparent neuronal and glial properties of these cells (Davidoff *et al.* 2004). Before an understanding of adult Leydig cell differentiation and

development can be achieved, the origin of the cells needs to be established and further studies are needed.

A convenient model for the investigation of adult Leydig cell development is the ethane dimethane sulfonate (EDS)-treated rat. In the adult animal, a single injection of EDS leads to rapid ablation of the existing adult Leydig cells followed by Leydig cell repopulation in a manner that appears to recapitulate the normal developmental process (Teerds 1996, Ariyaratne *et al.* 2003). This model, therefore, offers the possibility for studying Leydig cell differentiation in a relatively stable environment without the other ongoing background developmental processes that occur naturally as the animal grows through puberty. A potential problem of the EDS model, however, is that loss of Leydig cells and, therefore, androgen secretion is rapidly followed by atrophy of the androgen-dependent spermatogenic epithelium, which repopulates as the Leydig cells re-appear (Sharpe *et al.* 1990). This, of itself, is likely to have a marked effect on sertoli cell activity, which will confound studies on Leydig cell re-generation. In an attempt to circumvent this problem, we have adapted a model of EDS-induced Leydig cell depletion which used germ cell-free animals (Molenaar *et al.* 1986). Clearly, this will avoid problems of germ cell depletion and re-generation following EDS. In addition, Leydig cell repopulation occurs more rapidly in rats in which the spermatogenic epithelium is absent either experimentally (Molenaar *et al.* 1986) or developmentally (Edwards *et al.* 1988a). This shortening of the re-generation process is likely to increase the chances that transient changes in gene expression will be detected. We report here on the characteristics of Leydig cell depletion and re-generation and the expression of cell signaling molecules following EDS treatment in germ cell-depleted testes.

Results

Effects of busulfan and EDS on testis morphology and androgen levels

The busulfan regime used in this study caused a decline in testis weight from 1.96 ± 0.09 to 0.77 ± 0.02 g 6 weeks after the second injection. This was associated with depletion of germ cells so that tubules were largely devoid of germ cells at the start of EDS treatment (Fig. 1A and B). The intratubular nuclei remaining after busulfan treatment had the characteristics of Sertoli cell nuclei (Fig. 1B). The Leydig cells appeared densely packed in the interstitium after germ cell depletion because of tubular shrinkage (Fig. 1A and B).

Treatment with EDS caused a further decline in testis weight over the next 8 days followed by a recovery up to 20 days (Fig. 2A). Testosterone levels dropped markedly over the first 24 h following EDS and reached a nadir at 3–5 days before starting to recover at 8 days. By 20 days the levels of testosterone were about 25% of controls (Fig. 2B). Leydig cell numbers were depleted 24 h after EDS, and by 3 days post-treatment were almost completely ablated

with only a very occasional, morphologically distinct, Leydig cell apparent in the interstitial space (Fig. 1C).

Immunohistochemical expression of CYP11A1

In untreated rats and in busulfan-treated rats prior to EDS, there was strong expression of cytochrome P450 11a1 (CYP11A1, a marker of steroidogenic cells) in the interstitial Leydig cells. This staining was no longer present by 3 days after EDS (Fig. 1A–C). By 8 days after EDS treatment staining for CYP11A1 had started to re-appear in some peritubular cells and remained exclusively associated with the peritubular cells up to 20 days (Fig. 1D–F). At 8 days there were no CYP11A1-positive cells developing next to blood vessels, which were not also lying in a peritubular position. At day 20 the immunostained cells mainly formed clumps closely opposed to the external surface of the tubule although some cells appeared within the interstitial space (Fig. 1F).

Testicular mRNA expression levels following EDS

Leydig cell-specific genes

Levels of mRNA encoding the three steroidogenic enzymes CYP11A1, cytochrome P450 17a1 (CYP17A1) and 3 β -hydroxysteroid dehydrogenase type 1 (HSD3B1), and the luteinizing hormone receptor (LHR) showed an expression profile after EDS which closely resembled changes in intratesticular testosterone (Fig. 3). There was a rapid fall in expression 24 h after treatment reaching a nadir around 3–5 days with recovery starting on day 8. The notable difference to testosterone levels is that expression of these genes returned to normal by 20 days whereas testosterone was still reduced by 75% at 20 days. The other major steroidogenic enzyme specific to the Leydig cells is 17 β -hydroxysteroid dehydrogenase (HSD17B3), and levels of *Hsd17b3* mRNA declined rapidly after EDS to become undetectable by 3 days. Unlike the other steroidogenic enzymes, however, levels of *Hsd17b3* showed no recovery of expression up to day 20. The levels of *Pdgfra* mRNA declined about 75% after EDS and remained low up to 20 days.

Cell-signaling molecules

Within 24 h of EDS treatment there was a marked, sixfold increase in *Pdgfra* mRNA transcript levels (Fig. 4). This change in expression was transient and levels returned to normal by 3 days. There was a similar, though smaller, increase in levels of *Lif* after EDS. In contrast, levels of *Dhh* mRNA transcripts declined by about 80% within the first 24 h of EDS treatment then returned to normal by 3 days, while insulin-like growth factor I (*Igf1*) levels declined to 30% of normal at 24 h and remained low up to 5 days. Other signaling molecules measured

Figure 1 Immunohistochemical localization of CYP11A1 in the testis of untreated rats and rats treated with busulfan to destroy germ cells followed, 10 weeks later, by treatment with EDS. Testis sections (5 μ m) were incubated with antibody to CYP11A1 and visualized following binding to a biotinylated secondary antibody. In untreated animals (A) and animals treated only with busulfan (B) there was clear and abundant staining in the interstitial tissue. By 3 days after EDS (C) there was no staining for CYP11A1 but after 8 (D) and 12 (E) days clear staining was seen in peritubular cells (red arrows). By 20 days after EDS (F) groups of peritubular cells were stained (red arrows) and some of these cells were spreading into an interstitial localization. This figure also shows that the busulfan treatment regime significantly depleted the germ cell population (e.g., compare A and B). The bar in each picture represents 30 μ m.

(*Kitl* and wingless-related MMTV integration site 5A (*Wnt5a*)) showed no significant change in mRNA levels following EDS treatment.

Other genes

Levels of nuclear receptor subfamily 5a1 (*Nr5a1*, also known as *Sf1*) transcripts declined by about 50% immediately after EDS treatment but did not differ from control thereafter until 20 days when transcript levels were significantly increased (Fig. 5). Similarly, the levels of *Arx* transcripts declined immediately after EDS but were not significantly different to control thereafter (Fig. 5). Levels of neurofilament heavy polypeptide (*Nefh*) increased significantly 24 h after EDS treatment

and then returned to normal whilst there was no significant change in levels of nestin (*Nes*) transcripts.

Discussion

In the normal rat, the adult Leydig cell population begins to develop around day 12 after birth and reaches adult numbers by about day 40 (Ariyaratne & Chamindrani Mendis-Handagama 2000, Ariyaratne *et al.* 2000a). The underlying mechanism that initiates this process is unknown but recent studies have shown that the testicular interstitium of neonatal rats contains stem cells that can differentiate into steroidogenic cells *in vitro* or follow transplantation *in vivo* (Ge *et al.* 2006). Current

Figure 2 Testis weight (A) and total intratesticular testosterone (B) in rats treated with busulfan to destroy germ cells followed, 10 weeks later, by treatment with EDS. Data show changes in testicular weight and testosterone up to 20 days after EDS. Groups with different letter superscript are significantly different ($P<0.05$).

evidence suggests that Leydig cell re-population of the adult testis following EDS treatment is very similar to the normal developmental process (Teerds 1996, Ariyaratne *et al.* 2003) and it is likely that the same stem cells act as precursors of re-populating Leydig cells. The EDS model offers, therefore, a convenient method for the study of Leydig cell development while use of germ cell-free animals, as described here, allows this to be done on a relatively stable background with no other on-going, age-related, developmental events and without the massive degeneration and re-population of the germ cells which normally occurs following EDS.

Following EDS treatment, changes in the levels of mRNA species known to be specific to the Leydig cells followed, in the most part, changes in testosterone levels. The clear exception was *Hsd17b3* mRNA which declined at the same rate as other steroidogenic enzymes but did not show any recovery and remained undetectable up to 20 days. Failure of *Hsd17b3* expression to recover at the same rate as other enzymes may explain why testosterone levels remained relatively low at day 20. In an earlier study we showed that 17-ketoreductase activity (enzyme activity catalyzed by HSD17B3) declined more slowly after EDS than other steroidogenic enzyme activities and remained detectable at all times (O'Shaughnessy & Murphy 1991). This apparent discrepancy is probably due to the presence of other members of the 17 β -hydroxysteroid dehydrogenase family in the testis (Sha *et al.* 1996).

Figure 3 Testicular levels of mRNA species encoding Leydig cell-specific genes in rats treated with busulfan to destroy germ cells followed, 10 weeks later, by treatment with EDS. Data show changes in testicular transcript levels up to 20 days after EDS. Levels of mRNA were measured by real-time PCR and bars represent the mean $\pm$ S.E.M. of data from between three and five animals. Groups with different letter superscript are significantly different ($P<0.05$).

In the first study of EDS action on the germ cell-free testis it was reported that Leydig cell repopulation after EDS was faster than in normal rats (Molenaar *et al.* 1986). This is confirmed by our current study which shows that levels of testosterone and most Leydig cell mRNA markers began to recover by 8 days after EDS treatment. This contrasts with normal rats in which testosterone levels begin to recover between 14 and 21 days after EDS (Molenaar *et al.* 1986, Edwards *et al.* 1988b, Sharpe *et al.* 1990, Ariyaratne *et al.* 2003). It is known that germ cell depletion affects Sertoli cell function (McKinnell & Sharpe 1997, Guitton *et al.* 2000, O'Shaughnessy *et al.* 2008) and so the accelerated re-generation of active Leydig cells in germ cell-depleted testes may be due to altered Sertoli cell activity. Alternatively, LH is required for re-generation of Leydig cells after EDS treatment (Molenaar *et al.* 1986, Teerds *et al.* 1989) and levels of LH are higher in germ cell-free animals treated with EDS compared with normal EDS-treated animals (Morris & Jackson 1978, Molenaar *et al.* 1986). The more rapid Leydig cell re-generation in germ cell-free animals may, therefore, reflect increased tropic stimulation of the developing precursor cells.

Previous studies of adult Leydig cell differentiation, either during normal development or following EDS

Figure 4 Testicular levels of mRNA species encoding cell-signaling molecules in rats treated with busulfan to destroy germ cells followed, 10 weeks later, by treatment with EDS. Data show changes in testicular transcript levels up to 20 days after EDS. Levels of mRNA were measured by real-time PCR and bars represent the mean $\pm$ S.E.M. of data from between three and five animals. Groups with different letter superscript are significantly different ($P < 0.05$). If no superscripts are shown there was no difference between groups.

in the adult, have identified peritubular, spindle-shaped/mesenchymal cells as the immediate precursor cells (Haider *et al.* 1995, Russell *et al.* 1995, Ariyaratne *et al.* 2000a, 2000b). In addition, spindle-shaped peritubular cells have been described recently as Leydig cell stem cells (Ge *et al.* 2006). In contrast, it has recently been proposed that adult Leydig cells differentiate from perivascular smooth muscle cells and pericytes (Davidoff *et al.* 2004). This is based on the hypothesis that Leydig cells show neural characteristics and that cells expressing *Nes*, which is a stem cell marker in the nervous system, appear to differentiate from the perivascular region of the testis after EDS and may then go on to form adult Leydig cells (Davidoff *et al.* 2004). In addition, expression of *Nefh*, an intermediate filament protein expressed in the neuronal/glial lineage subsequent to *Nes*, also increases transiently in perivascular cells. Our studies agree that there is an increase in testicular *Nefh* expression following EDS although no significant change in *Nes* was seen. More crucially, however, we saw no evidence of perivascular development of Leydig cells. All cells expressing CYP11A1 during the initial phases of differentiation (around 8 days) were peritubular. From this evidence we cannot rule out a perivascular origin for Leydig cells, and there are other studies suggesting a minor contribution from this source

Figure 5 Testicular levels *Nr5a1*, *Arx*, *Nefh* and *Nes* mRNA in rats treated with busulfan to destroy germ cells followed, 10 weeks later, by treatment with EDS. Data show changes in testicular transcript levels up to 20 days after EDS. Levels of mRNA were measured by real-time PCR and bars represent the mean $\pm$ S.E.M. of data from between three and five animals. Groups with different letter superscript are significantly different ($P < 0.05$). If no superscripts are shown there was no difference between groups.

(Haider *et al.* 1995, Haider & Servos 1998), but it is unlikely to be the major site of Leydig cell precursor differentiation.

PDGF is a major mitogen for mesenchymal cells and there is a growing body of evidence that links PDGF signaling with Leydig cell differentiation and development. In *Pdgfra*-null mice, for example, adult Leydig cells fail to develop, possibly because of a failure of precursor proliferation (Gnessi *et al.* 2000). Consistent with this hypothesis, the putative Leydig cell stem cells express *Pdgfra* and PDGFA stimulates stem cell proliferation (Ge *et al.* 2006). The early, transient rise in *Pdgfra* mRNA levels after EDS treatment is, therefore, of considerable interest and would be consistent with a role for PDGFA in Leydig cell precursor proliferation and differentiation. In addition to PDGFA, the myoid cell factor LIF also stimulates Leydig cell stem cell proliferation (Ge *et al.* 2006). Levels of *Lif* mRNA increased in the testis 24 h after EDS treatment and then returned to basal levels within 3–5 days. In embryonic stem cells LIF withdrawal is a stimulus for differentiation (Ward *et al.* 2004) and it is possible that the transient change in levels of *Lif* after EDS acts both to enhance stem cell proliferation and then to permit Leydig cell differentiation.

DHH is a cell signaling molecule produced by the sertoli cells and essential for normal differentiation of the fetal and adult Leydig cell populations (Clark *et al.* 2000, Yao *et al.* 2002). In DHH-null mice, there is failure of adult Leydig cell differentiation although this may be through failure of precursor proliferation or development (Clark *et al.* 2000). Following EDS treatment levels of mRNA encoding, DHH declined transiently but

significantly at 24 h. It is not clear whether this may be related to subsequent Leydig cell re-generation or is simply a response to declining testosterone levels. Levels of *Igf1* mRNA also declined after EDS although, unlike *Dhh*, levels remained significantly reduced up to 8 days. The pattern of *Igf1* expression after EDS is consistent with partial expression in the Leydig cells as reported previously (Moore *et al.* 1993).

This study characterizes a model system with which to study Leydig cell degeneration and re-generation without marked changes in the germ cell population. Leydig cell re-generation is also more rapid in this system, which may make it simpler to identify changes in gene expression associated with these changes. Our results also provide further support for a peritubular origin of adult Leydig cells and suggest that increasing levels of PDGFA and LIF following Leydig cell ablation may contribute to precursor proliferation.

Materials and Methods

Animal treatments

Adult Sprague–Dawley rats (350–400 g) were purchased from Harlan UK (Bicester, Oxfordshire, UK). To remove the germ cell population in these animals they were treated with the cytotoxic drug busulfan that selectively ablates spermatogonia

(Jackson *et al.* 1962). Rats are sensitive to the toxic effects of busulfan, and initial studies were designed to identify a treatment regime that was effective in ablating the germ cell population without clear systemic toxicity. The most effective regime identified was a modification of one described earlier (Jiang 1998) and required two injections (i.p.) of busulfan (10 mg/kg) spaced 4 weeks apart. Under this regime, daily observations of the animals indicated that there was no gross toxicity. Six weeks after the second injection of busulfan the animals were injected (i.p.) with EDS (100 mg/kg) or vehicle. Animals were killed by CO₂ inhalation at different times after EDS treatment. One testis was processed immediately for RNA extraction while the other was weighed, cut into two halves and one half fixed in Bouin's medium while the other half was frozen rapidly in liquid N₂ and stored at –20 °C.

Gene expression studies

Total RNA was extracted from one testis of each animal by homogenization in TRizol (Invitrogen Ltd). At the start of the extraction process, luciferase mRNA (5 ng) was added to each sample to act as an external standard for real-time PCR (Baker & O'Shaughnessy 2001). Extracted RNA was reverse transcribed using random hexamers and Moloney murine leukemia virus reverse transcriptase (Superscript II, Invitrogen Ltd) as described previously (O'Shaughnessy & Murphy 1993, O'Shaughnessy *et al.* 1994). Levels of mRNA were measured

Table 1 Primers used for real-time PCR.

Gene	Abbreviation	Primers
Cytochrome P450 side chain cleavage	<i>Cyp11a1</i>	GCAGCGACTCTCTCTCCTGCG GCCATCACCTCTTGGTTAGGACAATT
3 β -hydroxysteroid dehydrogenase ^a	<i>Hsd3b1</i>	GACAGGAGCAGGAGGGTTTGTGG CTCCTTCTAACATTGTACCTTGGCCT
Luteinising hormone receptor	<i>Lhr</i>	CTGCGCCTTCAGGAATTTGCC AATCATAATCCCAGCCACTGAGTTCATTCT
Cytochrome P450 17 α -hydroxylase	<i>Cyp17a1</i>	TGGTCCCATCTATTCTCTTCGCTCG AAGGCCACGCCTTTCCCTTG
17 β -hydroxysteroid dehydrogenase type 3	<i>Hsd17b3</i>	AGTGTGTGAGGTTCTCCCGGTACCT TACAACATTGAGTCCATGTCTGGCCAG
Desert hedgehog	<i>Dhh</i>	CGTCCCCAACTACAACCCCG CGTCCCAACCTTCAGTCACGC
Wingless-related MMTV integration site 5A	<i>Wnt5a</i>	CGCGAAGACGGGCATCAAAG TGGCATTTACCACTCCAGCAGC
Platelet-derived growth factor α	<i>Pdgfa</i>	AGGTGAAGCGCTGCACTGGC TGGCACTCCAGGTGCTCT
Pdgf-receptor α	<i>Pdgfra</i>	GATAGCTTCATGAGCCGACACCCA CATTTGGCACGTACTGTGTGGTGTC
Kit ligand	<i>Kitl</i>	CCGGGATGGATTGTTTGCCTAGTC CACGAGGTCATCCACTATTTCCCAA
Insulin-like growth factor 1	<i>Igf1</i>	TGACCTGCTGTGTAACGACCCG CAGCTCAGCCCCGCAAAGG
Leukemia inhibitory factor	<i>Lif</i>	GCCATACGCCACCCGTGTCA TCCGTCATGTTTGGCGACA
Neurofilament heavy polypeptide	<i>Nefh</i>	TCGAAGGACACCGGTGCAGAGTA CCAACTCTGTGGTCTGGCCTG
Nestin	<i>Nes</i>	AGAAGCGCTGGAACAGAGATTGGA TCTTGAGG TGTGCCAGTTGCTGC
Arx homeobox	<i>Arx</i>	GTTACCGCACCACGTTACACAGTTA CCACTTGGCCCTGCGGTTCT
Nuclear receptor 5a1	<i>Nr5a1</i>	CAGGATGGCGGACCAGACCTTTAT CCTTGCCGTACTGGACCTGGC

^aAmplifies both type1 and type2 isoforms.

by real-time PCR using the SYBR green method with a Stratagene MX3000 cycloer. Reactions contained 5 µl 2× SYBR mastermix (Stratagene, Amsterdam), primers (100 nM), and template in a total volume of 10 µl. The thermal profile used for amplification was 95 °C for 8 min followed by 40 cycles of 95 °C for 25 s, 63 °C for 25 s, and 72 °C for 30 s. At the end of the amplification phase a melting curve analysis was carried out on the products formed. All primers were designed by Primer Express 2.0 (Applied Biosystems, Warrington, UK) using parameters previously described (Czechowski *et al.* 2004). The primers used are described in Table 1.

Immunohistochemistry

Testes were fixed overnight in Bouin's medium and stored in 70% ethanol. Wax sections (5 µm) were incubated with primary antibody overnight at 4 °C and endogenous biotin was blocked using an avidin/biotin blocking kit (R&D systems Europe Ltd, Abingden, UK). The antibody used was rabbit anti-bovine CYP11A1 (gift from A H Payne). Sections were washed and incubated for 30 min with biotinylated secondary antibody (R&D Systems Europe Ltd, Abingden, UK). Bound anti-body was visualized using 3,3'-diaminobenzidine tetrahydrochloride (R&D Systems Europe Ltd). Negative controls without the primary antibody were included in each experiment.

RIA

To measure total intratesticular testosterone, steroids were extracted from the testes with ethanol and measured by RIA as previously described (Sheffield & O'Shaughnessy 1989).

Statistical analysis

Data were analyzed by single-factor ANOVA followed by Fisher's multiple comparison test (Minitab, Minitab Ltd, Coventry, UK). Where necessary, data were log-transformed before analysis to avoid heterogeneity of variance.

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References

- Ariyaratne HB & Chamindrani Mendis-Handagama S 2000 Changes in the testis interstitium of Sprague-Dawley rats from birth to sexual maturity. *Biology of Reproduction* **62** 680–690.
- Ariyaratne HB, Mendis-Handagama SM, Hales DB & Mason JI 2000a Studies of the onset of Leydig precursor cell differentiation in the prepubertal rat testis. *Biology of Reproduction* **63** 165–171.
- Ariyaratne HB, Mills N, Mason JI & Mendis-Handagama SM 2000b Effects of thyroid hormone on Leydig cell regeneration in the adult rat following ethane dimethane sulphonate treatment. *Biology of Reproduction* **63** 1115–1123.
- Ariyaratne S, Kim I, Mills N, Mason I & Mendis-Handagama C 2003 Effects of ethane dimethane sulphonate on the functional structure of the adult rat testis. *Archives of Andrology* **49** 313–326.
- Baker PJ & O'Shaughnessy PJ 2001 Expression of prostaglandin D synthetase during development in the mouse testis. *Reproduction* **122** 553–559.
- Brennan J, Tilmann C & Capel B 2003 Pdgfr- α mediates testis cord organization and fetal Leydig cell development in the XY gonad. *Genes and Development* **17** 800–810.
- Clark AM, Garland KK & Russell LD 2000 Desert hedgehog (Dhh) gene is required in the mouse testis for formation of adult-type Leydig cells and normal development of peritubular cells and seminiferous tubules. *Biology of Reproduction* **63** 1825–1838.
- Czechowski T, Bari RP, Stitt M, Scheible WR & Udvardi MK 2004 Real-time RT-PCR profiling of over 1400 Arabidopsis transcription factors: unprecedented sensitivity reveals novel root- and shoot-specific genes. *Plant Journal* **38** 366–379.
- Davidoff MS, Middendorff R, Enikolopov G, Riethmacher D, Holstein AF & Muller D 2004 Progenitor cells of the testosterone-producing Leydig cells revealed. *Journal of Cell Biology* **167** 935–944.
- Edwards G, Lendon RG & Morris ID 1988a Accelerated recovery of Leydig cells of the immature rat testis after administration of the cytotoxic ethylene dimethanesulphonate. *Journal of Endocrinology* **119** 475–482.
- Edwards G, Robertson WR & Morris ID 1988b Characterization of the regenerated Leydig cell population of the rat after destruction by ethylene-1,2-dimethanesulphonate. *Journal of Endocrinology* **117** 11–18.
- Ge RS, Dong Q, Sottas CM, Papadopoulos V, Zirkin BR & Hardy MP 2006 In search of rat stem Leydig cells: identification, isolation, and lineage-specific development. *PNAS* **103** 2719–2724.
- Gnessi L, Basciani S, Mariani S, Arizzi M, Spera G, Wang C, Bondjers C, Karlsson L & Betsholtz C 2000 Leydig cell loss and spermatogenic arrest in platelet-derived growth factor (PDGF)-A-deficient mice. *Journal of Cell Biology* **149** 1019–1026.
- Guitton N, Touzalin AM, Sharpe RM, Cheng CY, Pinon-Lataillade G, Meritte H, Chenal C & Jegou B 2000 Regulatory influence of germ cells on sertoli cell function in the pre-pubertal rat after acute irradiation of the testis. *International Journal of Andrology* **23** 332–339.
- Haider SG & Servos G 1998 Ultracytochemistry of 3 β -hydroxysteroid dehydrogenase in Leydig cell precursors and vascular endothelial cells of the postnatal rat testis. *Anatomy and Embryology* **198** 101–110.
- Haider SG, Laue D, Schwochau G & Hilscher B 1995 Morphological studies on the origin of adult-type Leydig cells in rat testis. *Italian Journal of Anatomy and Embryology* **100** 535–541.
- Jackson JA, Partington M & Fox BW 1962 Effect of busulphan (Myleran) on the spermatogenic cell population of the rat testis. *Nature* **194** 1184–1185.
- Jiang FX 1998 Behaviour of spermatogonia following recovery from busulfan treatment in the rat. *Anatomy and Embryology* **198** 53–61.
- Kitamura K, Yanazawa M, Sugiyama N, Miura H, Iizuka-Kogo A, Kusaka M, Omichi K, Suzuki R, Kato-Fukui Y, Kamiirisa K *et al.* 2002 Mutation of ARX causes abnormal development of forebrain and testes in mice and X-linked lissencephaly with abnormal genitalia in humans. *Nature Genetics* **32** 359–369.
- Klonisch T, Fowler PA & Hombach-Klonisch S 2004 Molecular and genetic regulation of testis descent and external genitalia development. *Developmental Biology* **270** 1–18.
- Majdic G, Saunders PK & Teerds KJ 1998 Immunoeexpression of the steroidogenic enzymes 3- β hydroxysteroid dehydrogenase and 17 α -hydroxylase, C17,20 lyase and the receptor for luteinizing hormone (LH) in the fetal rat testis suggests that the onset of Leydig cell steroid production is independent of LH action. *Biology of Reproduction* **58** 520–525.
- McKinnell C & Sharpe RM 1997 Regulation of the secretion and synthesis of rat Sertoli cell SGP-1, SGP-2 and CP-2 by elongate spermatids. *International Journal of Andrology* **20** 171–179.
- Molenaar R, de Rooij DG, Rommerts FFG & van der Molen HJ 1986 Repopulation of Leydig cells in mature rats after selective destruction of the existent Leydig cells with ethylene dimethane sulphonate is dependent on luteinizing hormone and not follicle-stimulating hormone. *Endocrinology* **118** 2546–2554.

- Moore A, Chen CL, Davis JR & Morris ID** 1993 Insulin-like growth factor-I mRNA expression in the interstitial cells of the rat testis. *Journal of Molecular Endocrinology* **11** 319–324.
- Morris ID & Jackson CM** 1978 Gonadotrophin response after castration and selective destruction of the testicular interstitium in the normal and aspermatogenic rat. *Acta Endocrinologica* **88** 38–47.
- O'Shaughnessy PJ & Murphy L** 1991 Steroidogenic enzyme-activity in the rat testis following leydig-cell destruction by ethylene-1,2-dimethane-sulfonate and during subsequent leydig-cell regeneration. *Journal of Endocrinology* **131** 451–457.
- O'Shaughnessy PJ & Murphy L** 1993 Cytochrome P-450 17 α -hydroxylase protein and mRNA in the testis of the testicular feminized (Tfm) mouse. *Journal of Molecular Endocrinology* **11** 77–82.
- O'Shaughnessy PJ, Marsh P & Dudley K** 1994 Follicle-stimulating hormone receptor mRNA in the mouse ovary during post-natal development in the normal mouse and in the adult hypogonadal (hpg) mouse: structure of alternate transcripts. *Molecular and Cellular Endocrinology* **101** 197–201.
- O'Shaughnessy PJ, Hu L & Baker PJ** 2008 Effect of germ cell depletion on levels of specific mRNA transcripts in mouse Sertoli cells and Leydig cells. *Reproduction* **135** 839–850.
- Russell LD, De Franca LR, Hess R & Cooke P** 1995 Characteristics of mitotic cells in developing and adult testes with observations on cell lineages. *Tissue & Cell* **27** 105–128.
- Sha J, Baker P & O'Shaughnessy PJ** 1996 Both reductive forms of 17 β -hydroxysteroid dehydrogenase (types 1 and 3) are expressed during development in the mouse testis. *Biochemical and Biophysical Research Communications* **222** 90–94.
- Sharpe RM, Maddocks S & Kerr JB** 1990 Cell–cell interactions in the control of spermatogenesis as studied using Leydig cell destruction and testosterone replacement. *American Journal of Anatomy* **188** 3–20.
- Sheffield JW & O'Shaughnessy PJ** 1989 Effect of injection of gonadotropin-releasing hormone on testicular steroidogenesis in the hypogonadal (hpg) mouse. *Journal of Reproduction and Fertility* **86** 609–617.
- Teerds KJ** 1996 Regeneration of Leydig cells after depletion by EDS: a model for postnatal Leydig cell renewal. In *The Leydig Cell*, pp 203–220. Eds AH Payne, MP Hardy & LD Russell. Vienna: Cache River Press.
- Teerds KJ, de Rooij DG, Rommerts FFG, van den Hurk R & Wensing CJG** 1989 Proliferation and differentiation of possible Leydig cell precursors after destruction of the existing Leydig cells with ethane dimethane sulphonate: the role of LH/human chorionic gonadotrophin. *Journal of Endocrinology* **122** 689–696.
- Ward CM, Barrow KM & Stern PL** 2004 Significant variations in differentiation properties between independent mouse ES cell lines cultured under defined conditions. *Experimental Cell Research* **293** 229–238.
- Yao HH, Whoriskey W & Capel B** 2002 Desert Hedgehog/Patched 1 signaling specifies fetal Leydig cell fate in testis organogenesis. *Genes and Development* **16** 1433–1440.

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