Role of androgen and gonadotrophins in the development and function of the Sertoli cells and Leydig cells: data from mutant and genetically modified mice

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Summary

Development and maintenance of the male phenotype and establishment of fertility are all dependent upon the activity of the Sertoli cells and Leydig cells of the testis. This review examines the regulation and function of these cell during fetal and post-natal development. Fetal Leydig cells are sensitive to both luteinising hormone (LH) and adrenocorticotrophic hormone (ACTH) but Leydig cell function appears normal in fetal mice lacking both hormones or their receptors. Post-natally, the Sertoli cells and Leydig cells are reliant upon the pituitary gonadotrophins. Leydig cells are critically dependent on LH but follicle-stimulating hormone (FSH), presumably acting through the Sertoli cell, can also affect Leydig cell function. Testosterone secreted by the Leydig cells acts with FSH to stimulate Sertoli cell activity and spermatogenesis. Study of animals lacking FSH-receptors and androgen-receptors shows that both hormones can act to maintain the meiotic germ cell population but that androgens are critical for completion of meiosis.

Introduction

The testes are regulated by the pituitary gonadotrophins follicle-stimulating hormone (FSH) and luteinising hormone (LH). In the post-natal animal LH stimulates Leydig cells to secrete testosterone and both testosterone and FSH act to promote spermatogenesis through direct stimulation of the Sertoli cells. Developmental changes in circulating hormone levels and some of the major landmarks in testis development are shown in Fig 1. Most of this basic description of testicular regulation has been known since the 1920s and 1930s, through the pioneering work of Smith, Greep and others, (Smith and Engle, 1927; Greep and Fevold, 1937; Walsh et al., 1934). However, through the introduction of highly purified and recombinant gonadotrophins and, more recently, the generation of genetically modified mice (Huhtaniemi, 2006) we have been able to define more clearly both the role of these hormones and their mechanism of action at the cellular level. This article both reviews and presents novel data on the development and hormonal regulation of Leydig cell and Sertoli cell function. Our data relates specifically to the mouse but at the end of this article we have also tried to show how relevant the mouse is as a model for understanding control of testis development and function in the human.

Leydig cells

Fetal Leydig cells

Fetal Leydig cells first appear in the mouse at about 12.5 days post coitum (dpc), developing from mesenchymal-like stem cells within the interstitial space between the tubules (Byskov, 1986). Differentiation requires desert hedgehog (DHH), platelet-derived growth factor (PDGF) (possibly PDGFC) and expression of the X-linked Aristaless-related homeobox gene (*Arx*) (Pierucci-Alves et al., 2001; Yao et al., 2002; Brennan et al., 2003; Kitamura et al., 2002). DHH is secreted primarily by the Sertoli cells but *Pdgfc* is not of Sertoli cell origin and *Arx* expression is limited to peritubular myoid cells, endothelial cells and fibroblast-like cells in the fetal testis (Kitamura et al., 2002) showing that cells, other than the Sertoli cells, are involved in the differentiation process.

The fetal Leydig cells are essential regulators of masculinisation. Secretion of testosterone acts to rescue and stimulate growth of the male reproductive tract and external genitalia while a combination of testosterone and insulin-like growth factor 3 (INSL3) ensures appropriate testicular descent (Klonisch et al., 2004). In the mouse and rat it is not, however, clear how fetal Leydig cell function is regulated. Postnatal Leydig cells are critically dependent on LH and fetal Leydig cells respond to LH *in vitro* but Leydig cell function is normal in the fetus in the absence of LH or its receptor (O'Shaughnessy et al., 1998; Zhang et al., 2001b; Ma et al., 2004). Interestingly, in mice lacking a pituitary gland through a null-mutation in the *T/ebp* gene, testosterone levels are markedly reduced in late gestation indicating that for fetal Leydig cell function to be maintained in the normal range pituitary hormone support is essential (Pakarinen et al., 2002). Levels of pituitary-derived adrenocorticotrophic hormone (ACTH) are high in late fetal life and we have shown that fetal Leydig cells express the ACTH receptor (Johnston et al., 2007) and respond to ACTH *in vitro* with an increase in testosterone production (O'Shaughnessy et al., 2003). Study of mice lacking ACTH showed, however, that fetal testosterone levels were normal in these animals and that ACTH alone was not, therefore, responsible for fetal Leydig cell function (O'Shaughnessy et al., 2003). Nevertheless, the sensitivity of fetal Leydig cells to both LH and ACTH raised the possibility that both hormones may act, in a redundant fashion, to regulate fetal testosterone production *in vivo* (ie loss of one hormone or its receptor may be compensated by the presence of the other hormone). To test this hypothesis we generated mice lacking both ACTH

(Pomc-null) and the LH receptor (LuRKO) and measured testosterone levels in late fetal and early neonatal development (Fig. 2). Results showed that testosterone levels were normal in double knockout mice in late gestation and while there was reduced testosterone on the day of birth (day 1) this was not significantly different to LuRKO mice. This data indicates that LH and ACTH do not regulate fetal Leydig cell function and suggests either that another pituitary hormone is required or that the loss of fetal Leydig cell function in the *T/ebp*-null is not related to the loss of the pituitary and that other factors such as extra-pituitary hormones or paracrine factors may be involved. It is noteworthy that a number of putative paracrine factors have been shown to regulate the steroidogenesis of fetal, but not adult Leydig cells (El Gehani et al., 1998).

Adult Leydig cells

After birth, at about day 7 in the mouse, a second population of Leydig cells starts to differentiate (Baker et al., 1999; Nef et al., 2000). This "adult" population of Leydig cells gives rise to the pubertal increase in testosterone levels which is required for adult fertility. Unlike the fetal Leydig cell population, the factors regulating adult Leydig cell differentiation remain largely unknown. In the absence of DHH and PDGFA adult Leydig cells fail to develop but this may be related to a failure of stem cell development or proliferation (Clark et al., 2000; Gnessi et al., 2000; Park et al., 2007). It has been shown that the adult testis contains stem cells which will give rise to Leydig cells (Ge et al., 2006) but the origin of these cells and where they reside within the normal testis remains unknown. Surprisingly, the immediate progenitors of the adult Leydig cells also remain in some doubt. Earlier studies indicated that the adult Leydig cells differentiate from peritubular mesenchymal cells (Haider et al., 1995; Haider and Servos, 1998; Russell et al., 1995; Ariyaratne et al., 2000). A more recent study has reported, however, that Leydig cells develop from perivascular smooth muscle cells and pericytes during normal development or during Leydig cell regeneration following ablation with the cytotoxic drug ethane dimethane sulphonate (EDS) (Davidoff et al., 2004). To help clarify this apparent contradiction we examined expression of a specific marker of Leydig cells (CYP11A1) during the regeneration phase after EDS treatment in adult rats (O'Shaughnessy et al., 2008). In our studies all cells expressing CYP11A1 were in the peritubular region and we saw no evidence of perivascular development of Leydig cells (O'Shaughnessy et al., 2008). We cannot rule out a perivascular origin for Leydig cells from this evidence,

and there are other studies suggesting a minor contribution from this source (Haider et al., 1995; Haider and Servos, 1998), but it is unlikely to be the major site of Leydig cell precursor differentiation.

In contrast to the fetal Leydig cells, the adult Leydig cells are critically dependent on LH. There is evidence that the adult Leydig cell population will start to differentiate in the absence of LH (Baker et al., 2003; Zhang et al., 2004; Teerds et al., 2007) but Leydig cell numbers are reduced to about 10% of normal (Baker and O'Shaughnessy, 2001) and androgen production is barely detectable (O'Shaughnessy et al., 1998; Zhang et al., 2001a; Ma et al., 2004). Androgen also appears to be essential for adult Leydig cell function, at least in the mouse (Murphy et al., 1994; O'Shaughnessy et al., 2002; Eacker et al., 2008) and there is evidence, going back a number of years, that FSH may also play a role in Leydig cell function. The first data showed that purified or recombinant FSH would stimulate Leydig cell function in hypophysectomised rats (Chen et al., 1976; Kerr & Sharpe, 1985; Teerds et al., 1989; Vihko et al., 1991) and this has, since, been confirmed in the hypogonadal mouse (O'Shaughnessy et al., 1992; Sadate-Ngatchou et al., 2004a). In the testis, receptors for FSH are only found in the Sertoli cells (Heckert and Griswold, 2002) and the effects of FSH must be mediated by a factor or factors released by the Sertoli cells which act on the Leydig cells.

The EDS/Leydig cell regeneration model mentioned above is of use in studying regulation of Leydig cell development as the current evidence suggests that the regeneration process recapitulates normal development (Teerds & Rijntjes, 2007). We have, therefore, used real-time PCR to examine changes in mRNA transcript levels of specific signalling molecules during the early phase of Leydig cell regeneration (O'Shaughnessy et al., 2008). Results showed that there was a marked but transient increase in *Pdgfa* and *Leukemia inhibitory factor* (*Lif*) transcript levels 24h after treatment with EDS, at a time when Leydig cell ablation was nearly complete and regeneration starting (O'Shaughnessy et al., 2008). Both LIF and PDGFA are reported to stimulate Leydig cell stem cell proliferation (Ge et al., 2006), consistent with a role in Leydig cell regeneration after EDS. Surprisingly there was a significant decrease in *Dhh* levels 24h after EDS treatment which recovered by 48h (O'Shaughnessy et al., 2008). Static or declining levels of DHH after EDS treatment would suggest that DHH is not involved in the repopulation of adult Leydig cells although this requires further study. The EDS model of Leydig cell regeneration

offers significant advantages in the study of Leydig cell development, not least of which is the ability to isolate the event from other developmental changes which occur side by side with normal Leydig cell differentiation (Fig 1). Currently we do not know which signals (presumably from the Sertoli cells) trigger Leydig cell stem cell differentiation or, indeed, what stimulates the whole process and a more detailed analysis of transcriptional changes during this period is required.

Sertoli cells

Differentiation and fetal development

Differentiation of the Sertoli cells signals the start of testis differentiation at around 11.5dpc in the mouse. The Sertoli cells arise from precursors in the coelomic epithelium which covers the developing gonad; they then migrate inwards to form sex cords in the differentiating testis (Karl and Capel, 1998). The primordial germ cells (PGC) arise in the yolk sac and migrate to the gonad prior to differentiation. As testicular differentiation progresses the PGCs become embedded within the sex cords which enlarge to form the seminiferous tubules. Sertoli cell differentiation depends on SRY, SOX9 and NR5A1 (Sekido and Lovell-Badge, 2008) and since the Sertoli cell is the only cell type which requires the sex-determining gene SRY (Palmer and Burgoyne, 1991) it is likely that the Sertoli cell initiates at least the early stages of testicular differentiation. In addition, NR5A1 and SOX9 regulate the transcription of anti-Müllerian hormone (AMH) by the Sertoli cells leading to degeneration of the paramesonephric (Müllerian) ducts (de Santa et al., 1998).

The Sertoli cells proliferate during fetal and early neonatal testicular development in the mouse reaching their maximum adult numbers around post natal days 15-20 (Vergouwen et al., 1993; Baker and O'Shaughnessy, 2001). Like the fetal Leydig cells, the fetal Sertoli cells do not appear to be dependent upon direct hormonal stimulation. Sertoli cell number is normal during fetal development in *hpg* mice which lack gonadotrophin-releasing hormone (Baker and O'Shaughnessy, 2001) and in follicle-stimulating hormone (FSH) receptor-null (FSHRKO) mice (Johnston et al., 2004). In androgen-resistant *Tfm* mice Sertoli cell number is reduced on the day of birth (Johnston et al., 2004) but this is not through a direct effect on the Sertoli cells as they do not express androgen receptors (AR) at this time (You and Sar, 1998). Instead, it is highly likely that the effects of androgen are mediated through the peritubular myoid cells which are AR positive during fetal development (Tan et al.,

2005; You and Sar, 1998). Analysis of Sertoli cell transcript levels during development shows that the transcriptional activity in the cells is generally low during the fetal/neonatal proliferative phase and only increases once proliferation starts to slow down around day 10 (O'Shaughnessy et al., 2007). The clear exception to this generalisation being AMH which remains high throughout the proliferative phase (AlAttar et al., 1997). There is no evidence of androgen regulation of Sertoli cell transcript levels during the fetal period (O'Shaughnessy et al., 2007) but it is not currently known whether Sertoli cell function is normal during fetal development in FSHRKO mice.

Post-natal development

Post-natally, Sertoli cell function can be shown to be androgen- and FSH-sensitive by about days 5-6 (Johnston et al., 2004; O'Shaughnessy et al., 2007; Denolet et al., 2006), coinciding with the slowdown in Sertoli cell proliferation and general increase in cell activity. In adult FSHRKO mice Sertoli cell number is reduced but the animals are fertile (Dierich et al., 1998; Abel et al., 2000). In contrast, *Tfm* mice or mice lacking androgen receptors (AR) specifically in the Sertoli cells (SCARKO) are infertile due to a failure of spermatogenic progression beyond early meiosis (Lyon and Hawkes, 1970; De Gendt et al., 2004; Holdcraft and Braun, 2004; Chang et al., 2004). This highlights the importance of androgens in the regulation of Sertoli cell activity and a number of studies have used microarray technology to try to identify the major targets of androgen action in the Sertoli cell. These studies have used a variety of different models (androgen-treated normal (Zhou et al., 2005), androgen-treated *hpg* (Sadate-Ngatchou et al., 2004b), SCARKO (Denolet et al., 2006), *Tfm* (O'Shaughnessy et al., 2007) and AR hypomorph and Sertoli cell specific knockout mice (Eacker et al., 2007)) and the most notable characteristic of the data generated is how little consistency there is between the studies. The only transcript markedly altered by androgen action in all studies is *Rhox5* (formerly *Pem*) which is part of the reproductive homeobox gene cluster on the X chromosome (Maclean et al., 2005) and is known to be highly androgen sensitive (Lindsey and Wilkinson, 1996). The lack of consistency between array data is likely to be due to the different models used, to the age of the animals which varied from the neonate up to the adult and, possibly, to strain differences. These studies should, therefore, be seen as complementary rather

than contradictory and they suggest that androgen action may change as the Sertoli cell matures.

Despite differences between the array studies they point to at least three possible mechanisms of androgen action in the Sertoli cell, maintenance of cytoskeletal structure, metabolism of vitamin A and regulation of the Sertoli cell (blood-testis) barrier and tubular environment. In mice lacking androgen receptors there is misregulation of six transcripts (*Rims2*, *Bin3*, *Actn3*, *Cnn3*, *Nefh*, *Rai14*) associated with cytoskeletal structures and regulation of endocytosis and exocytosis (Eacker et al., 2007; O'Shaughnessy et al., 2007). In particular, four of these transcripts (*Bin3*, *Actn3*, *Cnn3*, and *Rai14*) have been linked to regulation of the actin cytoskeleton. Previous studies have shown that the actin cytoskeleton of the Sertoli cell and the actin-based junctional complexes involved in germ cell adhesion become disorganized after hypophysectomy and that this is prevented by androgen (Muffly et al., 1993), consistent with a role for androgens in regulating the Sertoli cell cytoskeleton. Vitamin A (retinol) is essential for male fertility, and deficiency leads to degeneration of spermatogenesis, probably through arrest of normal spermatogonial differentiation (Ghyselinck et al., 2006). Two studies have shown that transcripts involved in retinoic acid metabolism are regulated by androgen (O'Shaughnessy et al., 2007; Eacker et al., 2008) raising the possibility that there may be an interaction between androgen signaling and the retinoic acid pathway. In the normal testis tight junctions between Sertoli cells prevent movement of water-soluble factors into the lumen of the seminferous tubule and allows the Sertoli cells to generate a unique tubular environment for germ cell development. Data from the arrays and elsewhere suggest that this process may be androgen-dependent as androgens are required for the formation of the tight junctions and for normal expression of solute carriers (eg *Slc38a5*, *Slc39a8*, *Slc7a4*, *Slc4a8*, *Slc7a7*, *Slc25a10*, *Slc35a3*) (Meng et al., 2005; Fritz et al., 1983; O'Shaughnessy et al., 2007). Loss of androgens or androgen receptors may, therefore, disrupt spermatogenesis partly through changes in the tubular fluid composition.

Data from FSHRKO, *Tfm* and SCARKO mice show that both FSH and androgen are essential for normal Sertoli cell function and spermatogenesis. Androgens are clearly more critical, however, as spermatogenesis barely progresses beyond the pachytene spermatocyte stage in the SCARKO mouse and is even more severely disrupted in the *Tfm* mouse. What is less clear from these models, however,

is the degree of interaction and redundancy between the effects of FSH and androgen. To address this question we have generated mice lacking both AR and FSH receptors on the Sertoli cells (FSHRKO.SCARKO)(Abel et al., 2008). In these double knockouts Sertoli cell number was not different to FSHRKO mice but there was a marked reduction in total germ cell number compared to normal, FSHRKO and SCARKO mice. In addition, development of most cells stopped at the early pachytene stage with no secondary spermatocytes or round spermatids apparent (Fig 3) (Abel et al., 2008). Treating the FSHRKO.SCARKO mouse as a baseline control, the data indicates that the primary function of FSH is to increase Sertoli cell number and total germ cell number while androgens also act to increase germ cell number but, in addition, act to increase germ cell entry into meiosis and increase completion of meiosis. Together, the hormones have an additive effect on entry into meiosis and act synergistically to stimulate completion of meiosis and entry into spermiogenesis (Abel et al., 2008). These results are generally consistent with earlier studies based on hormone-replacement regimes (Russell et al., 1993; McLachlan et al., 1995; Singh et al., 1995; Haywood et al., 2003) but have the advantage that the FSHRKO.SCARKO mouse is a more clearly defined model of hormone/receptor deficiency and hormone action. Analysis of Sertoli cell-specific transcript levels in FSHRKO.SCARKO mice, compared to normal, FSHRKO and SCARKO mice, showed that about a third of the transcripts measured were independent of hormonal action on the Sertoli cell (Abel et al., 2008). Other transcripts measured were either predominantly androgen-dependent or showed redundant control by both FSH and androgen (Abel et al., 2008).

Overall, therefore, the data from FSHRKO.SCARKO mice indicates that both hormones can act to maintain the meiotic germ cell population but that androgens are critical for completion of meiosis. The Sertoli cell appears to retain a significant capacity for hormone-independent action but FSH and androgen act both independently and interactively to regulate levels of transcripts encoding factors critical for normal germ cell survival and progression.

Is the mouse a good model for human testicular development?

In general, mouse knockout models which are relevant to the development and regulation of testicular function provide reasonably accurate phenocopies of respective human mutations. Clearly there are no human equivalents of the cell-specific AR knockouts described above but the effects of complete AR inactivation on

spermatogenesis are similar in both mouse and human although the phenotype in the human may be more severe with few germ cells present and little progression into meiosis (Johnston et al., 2004; Hannema et al., 2006). In both species AR-null individuals are cryptorchid and the phenotype of AR-null testis is very similar to the cryptorchid testis (Johnston et al., 2004; Hedinger 1982). Differences between the AR-null mouse and human testis may, therefore, be at least a partial reflection of their sensitivity to elevated temperature. As discussed above, mouse *FSHβ* (Kumar et al., 1997) and *FSHR* (Abel et al., 2000; Dierich et al., 1998) knockout phenotypes are both fertile with slight reduction of sperm quality. Inactivating mutations of FSH*β* in humans cause azoospermia (Layman et al., 2002; Lindstedt et al., 1998; Phillip et al., 1998), but individuals with *FSHR* inactivation are oliozoospermic, and some have even sired children (Tapanainen et al., 1997). The exact human phenotype of FSH inactivation therefore remains unclear. The most conspicuous different between men and mice is the phenotype of LHR inactivation. *LHR* knockout mice are normally masculized at birth (Zhang et al., 2001a) whereas humans with completely inactivating LHR mutation are pseudohermaphrodites, totally or near-totally lacking masculinization *in utero* (Huhtaniemi and Themmen, 2005; Themmen and Huhtaniemi, 2000). Functional LHR in humal fetal Leydig cells is thus compulsory for their androgen synthesis, whereas in the mouse, as discussed above, paracrine or other endocrine factors must be able to maintain sufficient androgen synthesis by the fetal Leydig cells to induce masculinisation. The same species difference in fetal Leydig cell function is not, however, found in the case of *LH* inactivation (Lofrano-Porto et al., 2007; Valdes-Socin et al., 2004; Weiss et al., 1992; Ma et al., 2004)). In the human, placental hCG (an agonist of LH) can take over the function of missing LH while in the mouse, as we have shown, a number of different factors are able to compensate for missing LH. It is interesting that the two mammalian species have both developed backup mechanisms to compensate for missing LH action in the support of intrauterine masculinisation although the two mechanisms appear to be completely different.

Conclusions and future directions

The use of mutant and transgenic mice and the development of convenient, high throughput methods for measuring transcript and protein levels have significantly increased our understanding of testis development in recent years. A number of key

factors regulating testicular cell differentiation have been identified and the mechanisms underlying pituitary hormone and androgen control of testis development have become more established. There remain, of course, a number of outstanding gaps in our knowledge which need to be the targets of further study. For example, the nature and importance of interactions between the different cell types in the testis remains uncertain. Leydig cells regulate Sertoli cell function through secretion of androgens but how do Sertoli cells regulate Leydig cells and is this of physiological importance in the normal adult? What is the role of the peritubular myoid cells in testis development and function? How is fetal Leydig cell function controlled? These questions and others are likely to be answered in the near future by the increasing use and generation of mouse models which target gene ablation to specific cell types during testis development (eg Lecureuil et al., 2002).

Acknowledgements

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Figure Legends

Figure 1

Relative changes in circulating hormone levels during development in the male mouse. The approximate timing of landmark events in the development of the testis is shown by vertical arrows.

A Testis differentiation
B Fetal Leydig cell differentiation
C Spermatogonial differentiation
D Adult Leydig cell differentiation
E Onset of meiosis
F End of Sertoli cell proliferation
G Formation of Sertoli cell barrier
H Testis Descent
I Mature sperm present in testis

Figure 2

Intratesticular testosterone levels in normal (control), LuRKO, POMC-null (POMC) and LuRKO/POMC-null (LuRKO.POMC) mice. Intratesticular testosterone levels were measured on day 18 post coitum (A) and on post-natal day 1 (B). Results show the mean ± sem of 3 to 8 animals in each group. Two factor analysis of variance was used to analyse the data at each age. At post-coitum day 18 there was no difference between groups. At day 1 there was a significant effect of the LuRKO modification (* $P<0.05$) but no effect of POMC-null and no interaction.

Figure 3

Histology of teses from 8-wk-old normal, FSHRKO, SCARKO, and FSHRKO.SCARKO mice. Semi-thin sections stained with toluidine blue are shown and the bar represents 20μm. The tubules of normal and FSHRKO mice contained all stages of spermatogenesis although germ cell number was reduced in the FSHRKO mice. In SCARKO mice, spermatogenesis progressed through meiosis, but there was progressive loss of pachytene spermatocytes, and few secondary spermatocytes or round spermatids were observed. In FSHRKO.SCARKO mice, the tubular lumens were narrow and contained a high concentration of Sertoli cells (SC) with a smaller number of spermatogonia (S). Spermatogonia entered meiosis, but development stopped at early pachytene in most cells. Abundant Leydig cells (LC) were present within the interstitium.

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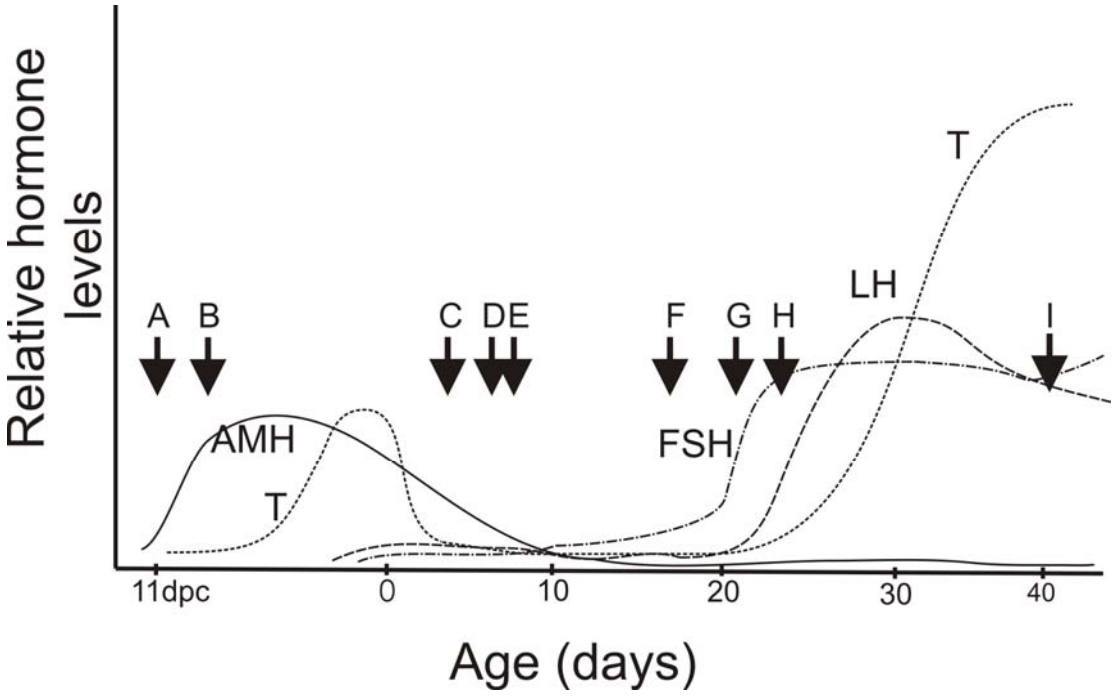
Figure 1:


Relative hormone levels
A
B
C
DE
F
G
H
I
AMH
T
FSH
LH
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11dpc
0
10
20
30
40
Age (days)

Figure 2:

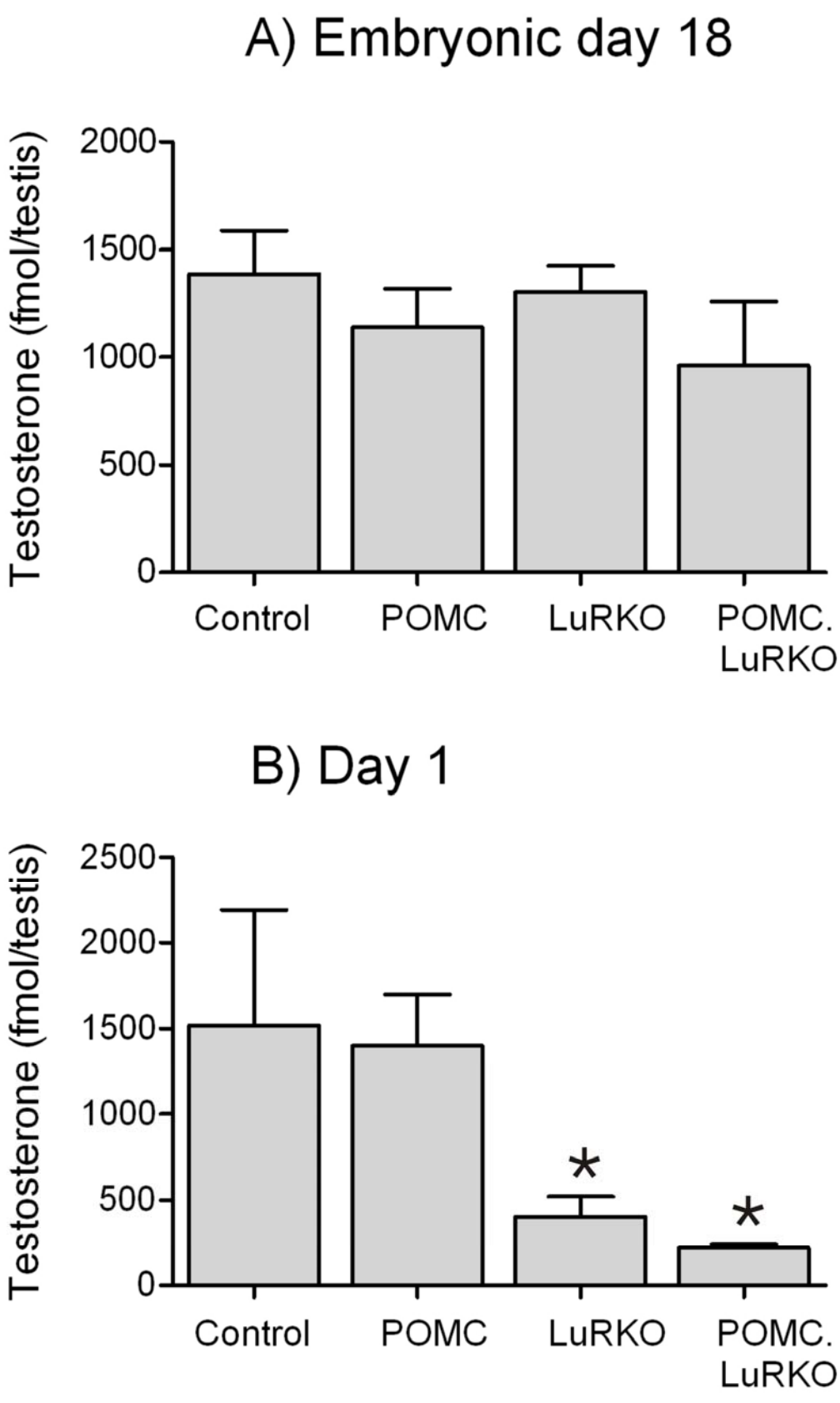
A) Embryonic day 18
Testosterone (fmol/testis)
2000
1500
1000
500
0
Control
POMC
LuRKO
POMC.
LuRKO
B) Day 1
Testosterone (fmol/testis)
2500
2000
1500
1000
500
0
*
*
Control
POMC
LuRKO
POMC.
LuRKO

Figure 3: