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Inhibin, activin, follistatin and FSH serum levels and testicular production are highly modulated during the first spermatogenic wave in mice

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Abstract

Testicular development is governed by the combined influence of hormones and proteins, including FSH, inhibins, activins and follistatin (FST). This study documents the expression of these proteins and their corresponding mRNAs, in testes and serum from mice aged 0 through 91 days *post partum* (dpp), using real-time PCR, *in situ* hybridisation, immunohistochemistry, ELISA and RIA. Serum immunoactive total inhibin and FSH levels were negatively correlated during development, with FSH levels rising and inhibin levels falling. Activin A production changed significantly during development, with subunit mRNA and protein levels declining rapidly after 4 dpp, while simultaneously levels of the activin antagonists, FST and inhibin/activin β_C, increased. Inhibin/activin β_A and β_B subunit mRNAs were detected in Sertoli, germ and Leydig cells throughout testis development, with the β_A subunit also detected in peritubular myoid cells. The α, β_A, β_B and β_C subunit proteins were detected in Sertoli and Leydig cells of developing and adult mouse testes. While β_A and β_B subunit proteins were observed in spermatogonia and spermatocytes in immature testes, β_C was localised to leptotene and zygotene spermatocytes in immature and adult testes. Nuclear β_A subunit protein was observed in primary spermatocytes and nuclear β_C subunit in gonocytes and round spermatids. The changing spatial and temporal distributions of inhibins and activins indicate that their modulated synthesis and action are important during onset of murine spermatogenesis. This study provides a foundation for evaluation of these proteins in mice with disturbed testicular development, enabling their role in normal and perturbed spermatogenesis to be more fully understood.

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Introduction

The first wave of spermatogenesis in the male mouse encompasses the proliferation and differentiation of germ cells and Sertoli cells over a period of 35 days (Fig. 1A). It is regulated by a complex interaction between circulating hormones, such as follicle-stimulating hormone (FSH) and luteinizing hormone (LH), and locally produced factors including activins, inhibins and androgens (Mather *et al*. 1997, de Kretser *et al*. 2001). Activin and inhibin are members of the transforming growth factor-β (TGF-β) superfamily that were initially identified by their ability to regulate FSH secretion at the pituitary. FSH regulates Sertoli cell function directly and germ cell development indirectly (reviewed in de Mather *et al*. 1997, de Kretser *et al*. 2001). Activins and inhibins are disulfide-linked dimeric proteins. The activins are homo- or heterodimers of the β subunits, β_A, β_B and β_C, encoded by *Inhba*, *Inhbb* and *Inhbc* respectively. The inhibins are heterodimers of an α subunit (encoded by *Inha*) and one of the same β subunits shared with the activins. The activin/inhibin nomenclature reflects the subunit composition of the proteins, and the following have been detected in mammalian cells and/or cell lines: activin A (β_A–β_A), activin B (β_B–β_B), activin AB (β_A–β_B), activin C (β_C–β_C), activin AC (β_A–β_C), inhibin A (α–β_A) and inhibin B (α–β_B) (Yu *et al*. 1994, Groome *et al*. 1996, Vihko *et al*. 1998, O'Connor *et al*. 1999, Mellor *et al*. 2000, 2003, Muttukrishna *et al*. 2000, Vejda *et al*. 2002).

The β_A subunit protein has previously been detected by immunohistochemistry in Sertoli cells, peritubular myoid cells, Leydig cells, gonocytes and endothelial cells in the testes of rats aged 3–9 days *post partum* (dpp; Meehan *et al*. 2000) and activin A is secreted by rat Sertoli and peritubular myoid cells *in vitro* (de Winter *et al*. 1993, 1994, Buzzard *et al*. 2003). The addition of activin A to cultures of rat testis fragments or cells enhances the FSH-mediated stimulation of immature Sertoli cell proliferation (Boitani *et al*. 1995, Meehan *et al*. 2000, Buzzard *et al*. 2003). Activin A increases gonocyte numbers in testis cultures from 3-day-old rats (Meehan *et al*. 2000) and inhibits the proliferative action of FSH on differentiating spermatogonia from 9 dpp rat testis cultures (Boitani *et al*. 1995). The mitogenic actions of activin on Sertoli cells are also evident from the observations of delayed fertility in

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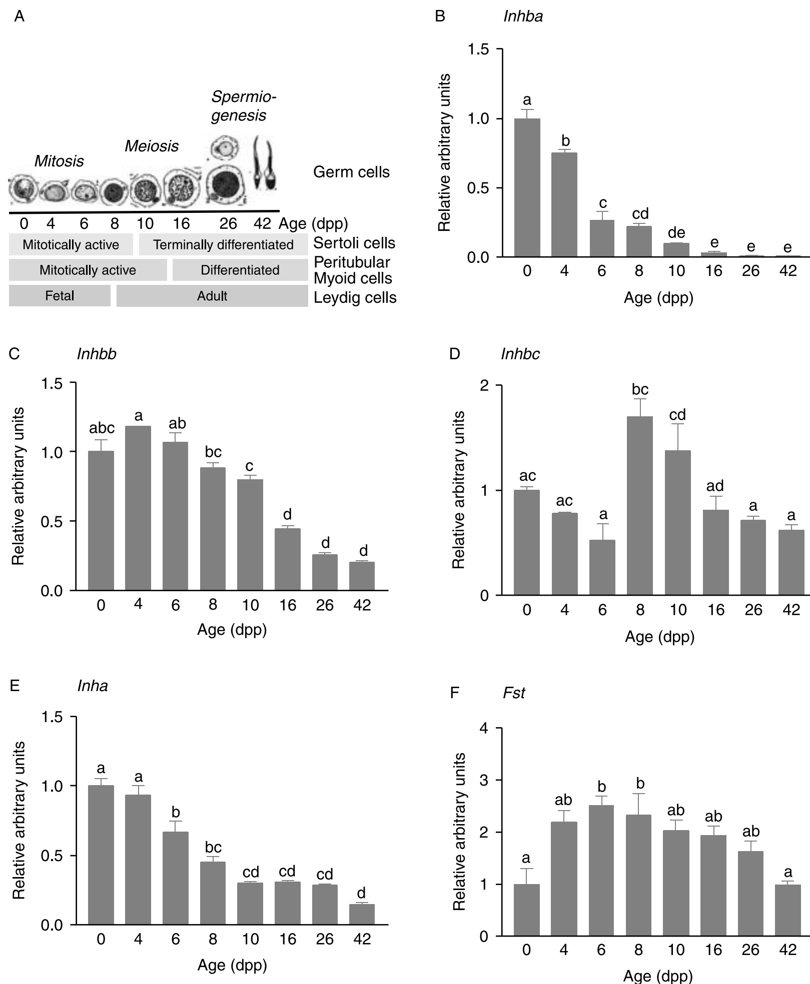

Figure 1 Development of the mouse testis and real-time PCR analysis of inhibin/activin subunits and follistatin mRNA. The first wave of murine spermatogenesis is depicted in (A). The most mature germ cells in the testis are represented at each age; gonocytes at 0 dpp, type A spermatogonia at 4 dpp, type B spermatogonia at 6 dpp, pre-leptotene spermatocytes at 8 dpp, zygotene spermatocytes at 10 dpp, pachytene spermatocytes at 16 dpp, round spermatids at 26 dpp and elongating spermatids at 42 dpp. Secondary spermatocytes are also seen from age 16 dpp onward (reviewed in de Rooij & Russell 2000; reviewed in McCarrey 1993; Orth 1993, Itman *et al.* 2006). Sertoli cells are mitotically active from before birth and then cease dividing and form Sertoli–Sertoli cell tight junctions between 7 and 11 dpp, at which point they are considered terminally differentiated. Pertibular myoid cells proliferate up to 14 dpp (Vergouwen *et al.* 1991). Leydig cells differentiate from fetal Leydig cells to adult Leydig cells at around 7 dpp (Vergouwen *et al.* 1993) and do not start to proliferate until after 21 dpp to reach adult levels by 35 dpp (Vergouwen *et al.* 1991). mRNA expression of (B) *Inhba*, (C) *Inhbb*, (D) *Inhbc*, (E) *Inha* and (F) *Fst* changes during testis development. Expression levels were analysed in triplicate on three different samples using real-time PCR and normalised relative to actin values. The values at each age are represented relative to the value at 0 dpp, which was arbitrarily set to 1. Different letters on each graph represent statistically significant data ($P<0.05$). Data are shown as mean ± s.e.m.

male mice, smaller testis size and reduced sperm output in mice following deletion of the activin type II receptor (Matzuk *et al.* 1995*b*) or replacement of the β_A subunit with the β_B subunit (Brown *et al.* 2000). Little is known about the specific role of activin B in testicular function, as mice in which the β_B subunit gene has been deleted do not exhibit a testicular phenotype (Vassalli *et al.* 1994). However, *in situ* hybridisation studies of adult human and rat testicular tissues revealed β_B subunit expression in Sertoli cells, Leydig cells and germ cells from spermatogonia to round spermatids (Marchetti *et al.* 2003, Buzzard *et al.* 2004), and immunoreactive β_B subunit was observed in human Leydig cells and pachytene spermatocytes and round spermatids (Marchetti *et al.* 2003).

The biological activity of activin is inhibited by many proteins, including follistatin (FST) and inhibin. There are two FST splice variants processed to form mature polypeptides of 288 (FST288) and 315 (FST315) amino acids. FST288 binds heparin sulphate proteoglycans and binds activins A and B, targeting them to an endocytotic degradation pathway (Hashimoto *et al.* 1997), while FST315 is the circulating form (Schneyer *et al.* 1994). Both isoforms bind activin with strong affinity making FST a potent antagonist of activin (Schneyer *et al.* 1994). Mice lacking the *Fst* gene die at birth (Matzuk *et al.* 1995*c*), so its precise role in post-natal testicular development has not been defined.

Mediated by their co-receptor, betaglycan, inhibins antagonise activin activity by directly binding to the activin type II receptors and thereby preventing type I receptor recruitment and activation in response to activin (Lewis *et al.* 2000, Wiater & Vale 2003). When cells simultaneously synthesize β subunits and α subunit, they have the potential to produce both inhibin and activin or both. Hence, the modulated production of either subunit has implications for both positive and negative effects on activin bioactivity.

Inhibin B is the major circulating inhibin in males and is produced by Sertoli and Leydig cells in the adult (de Kretser *et al.* 2001). The influence of inhibin B on adult spermatogenesis is not apparent, as mice that cannot produce inhibin B due to deletion of the gene encoding the β_B subunit are fertile (Vassalli *et al.* 1994, Kumar *et al.* 1999). However, the chronic absence of inhibin in the *Inha*$^{-/-}$ mouse (Matzuk *et al.* 1992) leads to development

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of stromal cell tumours by 4 weeks of age, due to the overproduction and unbalanced action of activin. Thus, inhibin is understood to play a critical role in the modulation of activin-induced Sertoli cell proliferation.

The function of the β_C subunit is also ambiguous as *Inhbc* null mice display no overt phenotype. However, transfection studies have shown that the β_C subunit forms heterodimers with the β_A subunit and thereby suppresses the formation of activin A homodimers, providing an additional pathway for intracellular control over activin A production and bioactivity (Mellor *et al.* 2003). The β_C subunit mRNA is expressed in adult human and rat testes (Loveland *et al.* 1996, Gold *et al.* 2004) and β_C subunit protein is detectable in the Sertoli and Leydig cells, spermatogonia, primary spermatocytes and elongating spermatids of the adult rat testis (Gold *et al.* 2004). However, there is currently no information about cellular sites or levels of β_C mRNA or protein expression during testis development.

Several groups, including ours, have examined the levels of inhibins, activins, FSH and FST during development of the rat testis (Au *et al.* 1986, Sharpe *et al.* 1999, Buzzard *et al.* 2004) and in humans at various ages (Massa *et al.* 1992, Anawalt *et al.* 1996, Andersson *et al.* 1997). The present study offers the first comprehensive expression analysis of these proteins during mouse testis development, and it provides data that highlight changes occurring at specific developmental stages of normal testis development. While using the best currently available assays, these data are limited by the absence of specific assays for mouse inhibin A and B and by the absence of reliable activin B assays. However, this report delineates discrete changes in the cellular sites of mRNA and protein synthesis that correspond to developmentally regulated production of activin and inhibin proteins. The data illustrate that their production is linked with dynamic circulating levels of these factors and of FSH, corresponding to key stages in germ cell development and testicular maturation.

Results

Levels of inhibin/activin subunits and Fst *mRNAs in the mouse testis*

The total testis *Inhba* mRNA levels (Fig. 1B) decline rapidly in the first week of spermatogenesis, with the level down by 75% at 6 compared with 0 dpp ($P<0.001$). *Inhba* mRNA levels continue to fall to 3% of the 0 dpp value ($P<0.001$) at 16 dpp and 0.4% ($P<0.001$) at 42 dpp (adulthood). In contrast, the *Inhbb* mRNA levels (Fig. 1C) remain high during the first week of spermatogenesis and decline after 10 dpp ($P<0.05$ between 10 and 16 dpp). This decline corresponds to the time when Sertoli cells become terminally differentiated and germ cells first enter meiosis. The *Inhbb* levels continue to decline to 25% of the 0 dpp level at 26 and 42 dpp ($P<0.001$).

The *Inhbc* mRNA levels (Fig. 1D) display a different profile to *Inhba* and *Inhbb*, with the lowest levels measured at 6 dpp followed by a 227% increase at 8 dpp ($P<0.001$). The *Inhbc* mRNA levels then decline significantly at 16 dpp ($P<0.01$ compared with 8 dpp) and remain low through to adulthood ($P<0.01$ between 8 and 26/42 dpp). The *Inha* mRNA values (Fig. 1E) show a progressive decline at 6, 8 and 10 dpp ($P<0.001$, between 6 and 10 dpp values) when levels are 30% of that measured at birth; they decline further to a nadir at 42 dpp, reaching an expression level that is 15% of the 0 dpp level ($P<0.001$).

Fst mRNA levels (Fig. 1F) exhibit a profile opposite to that of *Inhba*, with the level 119% higher ($P=0.053$) at 4 dpp and 150% higher at 6 dpp ($P<0.01$) compared with 0 dpp. *Fst* levels remain relatively high at subsequent ages to 26 dpp and decline to levels measured at birth at 42 dpp ($P<0.05$; comparing days 6–8 with day 42).

These data correlate well with information derived from Affymetrix microarrays in the GEO profile database (Small *et al.* 2005; http://www.ncbi.nlm.nih.gov/geo/).

Cellular localisation of mRNAs

Sertoli and Leydig cells contain mRNAs encoding both *Inhba* and *Inhbb* subunits from the time of birth through to adulthood (Fig. 2). Only *Inhba* is detected in the peritubular myoid cells throughout testicular development (Fig. 2A and B insets, C and D). At 0 dpp, the gonocytes do not contain detectable levels of either of the subunit mRNAs (Fig. 2A and I), but by 3 dpp, the spermatogonia display a signal for both subunit mRNAs, with the signal being more intense than that in Sertoli cells (Fig. 2B and J). At 15 dpp, the signal intensity observed for *Inhba* and *Inhbb* mRNAs in germ cells appears dependent on their state of maturation, with spermatogonia and pre-leptotene/leptotene spermatocytes showing a more intense signal than pachytene spermatocytes (Fig. 2C and K). In addition, the Sertoli cell *Inhbb* signal intensity varies between tubule cross-sections in accordance with the stage of the seminiferous cycle, and this pattern continues throughout testicular development to adulthood. The signal intensity is the greatest in stage VII Sertoli cells than at other stages (Fig. 2L). In the adult testis, both subunits are observed with the highest intensity in spermatogonia and pre-leptotene/leptotene spermatocytes and with progressively decreasing intensity in pachytene spermatocytes, round spermatids and elongating spermatids (Fig. 2D and L).

Cellular localisation of proteins

Immunohistochemistry revealed that, as with mRNA transcripts, the activin/inhibin β_A and β_B subunit proteins are detected in the Sertoli and Leydig cells throughout testicular development (Fig. 3). The β_c and inhibin α subunit

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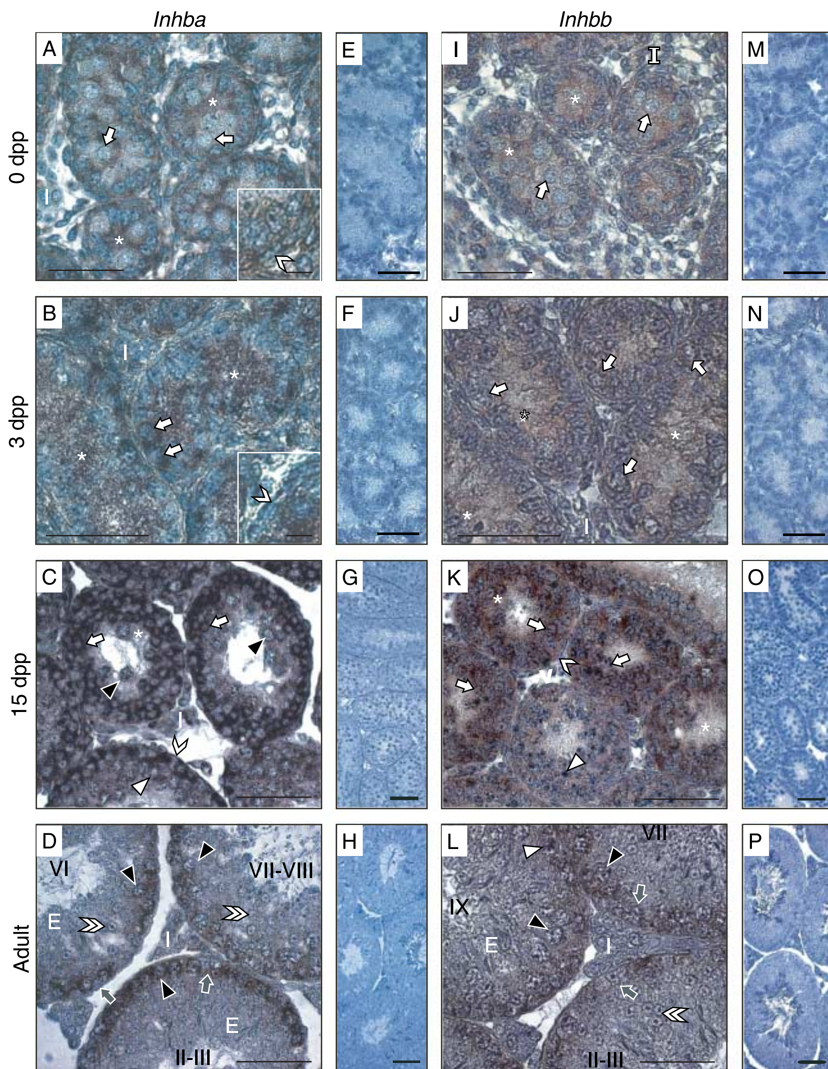

Figure 2 Cellular localisation of *Inhba* and *Inhbb* subunit mRNAs in mouse testes from birth to adulthood. Localisation using *in situ* hybridisation of the (A–D) *Inhba* and (I–L) *Inhbb* subunit mRNAs is shown at (A and I) 0 dpp, (B and J) 3 dpp, (C and K) 15 dpp and (D and L) adult ages. Both subunits are observed in the Sertoli cells, Leydig cells and spermatogonia at all ages, spermatocytes at 15 dpp, and spermatocytes and round spermatids at adult. No signal is observed with corresponding sense probes at all ages (negative controls for (E–H) *Inhba* and (M–P) *Inhbb*). The stages of the seminiferous epithelium are marked in the adult tubule cross-sections. All sections were counterstained with Harris haematoxylin. The asterisk symbol indicates the Sertoli cell cytoplasm (A–C and I–K); dark arrow points to Sertoli cell nucleus (D and L); white arrow points to gonocytes (A and I), spermatogonia (B and J) and pre-leptotene/-leptotene spermatocytes (C and K); white arrowhead points to zygotene spermatocytes (C–D and K–L); dark arrowhead points to pachytene spermatocytes (C–D and K–L); double arrow points to round spermatids (D and L); E indicates elongating spermatids (D and L); v-shaped arrowhead points to pertibular myoid cells; I indicates intertubular areas where Leydig cells are found. Scale bar represents 100 μm for A–D and I–L, 10 μm for A and C insets and 50 μm for negative control sections E–H and M–P.

proteins are also present in these cells, with the β_A and β_C subunit proteins also observed in peritubular myoid cells (Fig. 4). However, protein cellular expression in some germ cells differs to that observed for mRNAs. At 0 dpp, some gonocytes showed cytoplasmic localisation of the β_A subunit protein and at 15 dpp this subunit is detected in spermatogonia; however, this signal does not persist in spermatogonia of the adult (Fig. 3A–C). The β_A subunit protein is evident in the nucleus of leptotene and zygotene spermatocytes at 15 dpp (Fig. 3B, insets) and in zygotene and early pachytene spermatocytes at stages X–III of the adult testis, but this signal is less obvious in the pachytene and diplotene spermatocytes of stages IV–XI (Fig. 3C, insets).

The β_B subunit protein is detected in gonocytes at 0 dpp (Fig. 3D), and in spermatogonia and primary spermatocytes at 15 dpp (Fig. 3E), but not in the spermatogonia and spermatocyte populations of the adult testis (Fig. 3F). However, the β_B subunit protein is detected in elongating spermatids in adults (Fig. 3F). The negative controls relating to each protein detected are shown (Fig. 3G–I).

The inhibin α subunit protein was primarily detected in the Sertoli cells throughout post-natal testicular development. At 0 and 3 dpp, adjacent Sertoli cells differed in the inhibin α protein signal intensity (Fig. 4A), and at 15 dpp this differing signal intensity was also observed between tubule cross-sections (Fig. 4B). In the adult testis (Fig. 4C), the Sertoli cell inhibin α signal was stage-specific with highest intensity at stages X–III of the seminiferous epithelium cycle. The negative controls for each age are shown in Fig. 4D–F.

Immunolocalisation of the activin/inhibin β_C subunit protein was similar to that of the β_A subunit protein, with protein visualised in the cytoplasm of Leydig, Sertoli and peritubular myoid cells throughout testicular development (Fig. 4G–I). In germ cells at 0 dpp, the β_C subunit protein was localised to the nuclei of gonocytes (Fig. 4G) and, from 15 dpp to adulthood, it was detected only in the cytoplasm of pre-leptotene/leptotene and zygotene spermatocytes and in the nucleus of round spermatids (Fig. 4H and I) but was absent from elongating spermatids and spermatogonia

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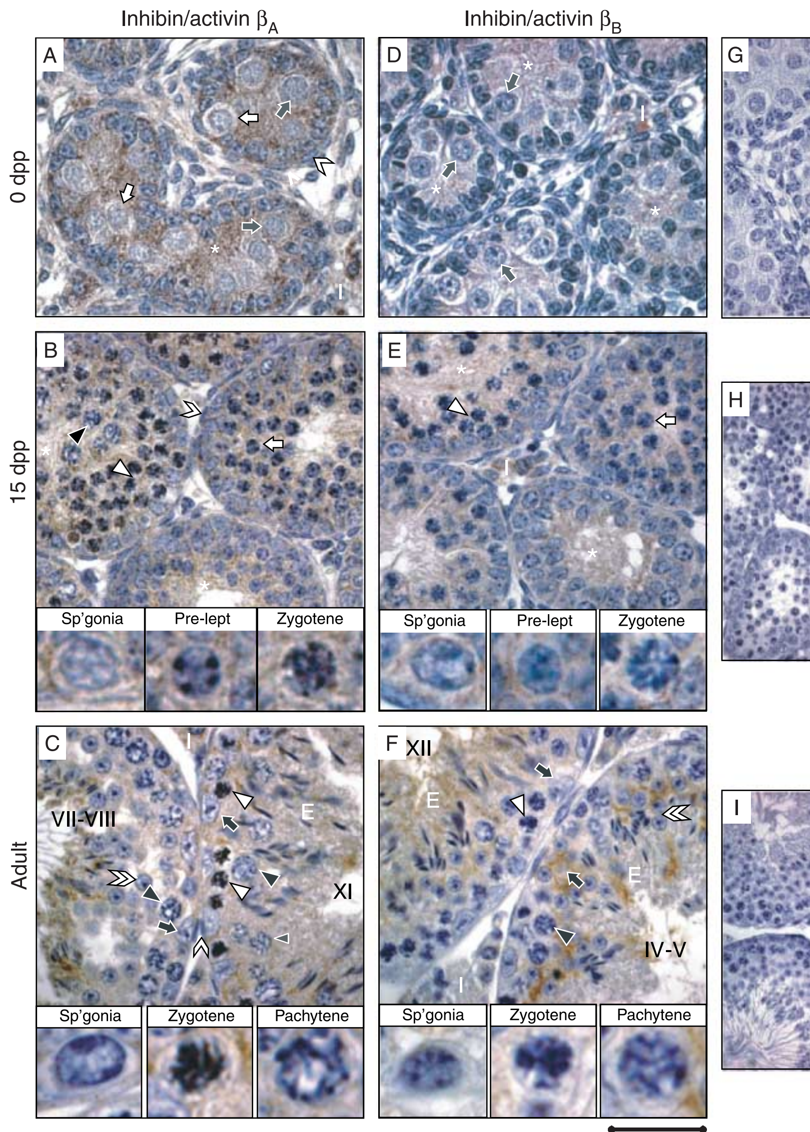

Figure 3 Immunolocalisation of inhibin/activin β_A and β_B subunits in mouse testes from birth to adulthood. Photomicrographs of mouse testes at ages (A and D) 0 dpp, (B and E) 15 dpp and (C and F) adult, immunostained for the inhibin/activin β_A (A–C) and β_B (D–F) subunits. Magnified images of a spermatogonia (Sp'gonia), pre-leptotene spermatocyte (Pre-lept) and zygotene spermatocyte from 15 dpp testes of are shown (B and E insets). Magnified images of a spermatogonia, zygotene spermatocyte and pachytene spermatocyte from adult testes are shown (C and F insets). The stages of the seminiferous epithelium are marked in the adult tubule cross-sections in C and F. G–I show the negative controls for each age relating to both proteins detected. All sections are counterstained with Harris haematoxylin. Asterisk symbol shows Sertoli cell cytoplasm (A, B, D and E); dark arrow points to Sertoli cell nucleus (C and F); white arrow points to gonocytes with negative signal and dark arrow points to gonocytes with positive signal (A and D); white arrow points to pre-leptotene/leptotene spermatocytes (B, C, E and F); white arrowhead points to zygotene spermatocytes (B, C, E and F); dark arrowhead points to pachytene spermatocytes (B, C, E and F); double arrow points to round spermatids (C and F); E indicates elongating spermatids (C and F); v-shaped arrowhead points to pertibular myoid cells; I indicates interstitial areas containing Leydig cells. Scale bar represents 50 μm for A–F, 80 μm for G–I and 12.5 μm for magnified images of individual cells.

(Fig. 4I). The negative controls for each age are shown in Fig. 4J–L. A summary of these cellular localisation data is provided in Table 1.

Testis weights

Testis weights increased progressively from 3.32±0.26 mg (paired weight) at birth to 189.8±4.2 mg (paired weight) at 91 dpp (Fig. 5A).

Testicular levels of activin, inhibin and FST

The concentrations of activin A, inhibin and FST per nanogram of testicular homogenate (Fig. 5B–D) provide a measure of their local concentrations, whereas the total testicular content takes into account the mass of the growing testis and provides an indication of the amount available for release into the circulation.

The testicular immunoactive inhibin concentrations rose significantly from 0 to 2 dpp ($P<0.05$) and peaked at 4 dpp (995.6±27.3 ng/g testis; Fig. 5B), corresponding to the period when gonocytes resume mitosis, migrate to the basement membrane and transform into spermatogonia. Thereafter, inhibin concentrations progressively declined to be significantly lower at 25 dpp ($P<0.05$ compared with 16 dpp) and plateau beyond day 45 dpp (248.2±15.52 ng/g testis). In contrast, total testis inhibin levels rose significantly from 1.07±0.09 ng/testis at 0 dpp to 25.5±2.1 ng/testis at 25 dpp ($P<0.001$) and remained stable thereafter.

The testicular concentrations of activin A (Fig. 5C) significantly declined from 0 to 1 dpp ($P<0.01$) before rising to a peak at 3 dpp (13.6±0.5 ng/g testis, $P<0.001$ compared with 1 and 2 dpp). Subsequently, the testis concentrations again declined by 8 dpp (3.4±0.4 ng/g testis, $P<0.001$ compared with each previous age) and

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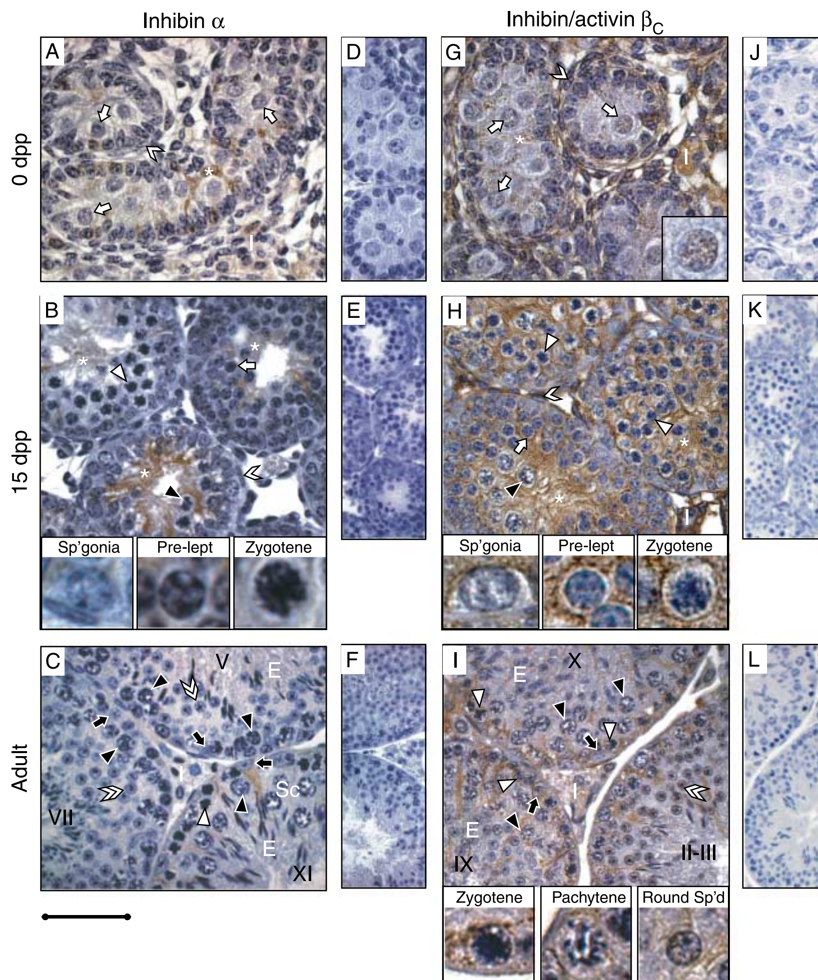

Figure 4 Immunolocalisation of inhibin α and inhibin/activin β_C subunits in mouse testes from birth to adulthood. Photomicrographs of mouse testes at ages (A and G) 0 dpp, (B and H) 15 dpp and (C and I) adult, immunostained for the (A–C) inhibin α and (G–I) inhibin/activin β_C subunits. Magnified images of a spermatogonia (Sp'gonia), pre-leptotene spermatocyte (Pre-lept) and zygotene spermatocyte from 15 dpp testes of B and H are shown (insets). Magnified images of a zygotene spermatocyte, pachytene spermatocyte and round spermatid from adult testes of I are shown (insets). The stages of the seminiferous epithelium are marked in the tubule cross-sections in C and I. D–F and J–L show the negative controls relating to inhibin α and inhibin/activin β_C subunits respectively. All sections are counterstained with Harris haematoxylin. Asterisk symbol shows Sertoli cell cytoplasm (A, B, G and H); dark arrow points to Sertoli cell nucleus (C and I); white arrow points to gonocytes (A and G); white arrow points to pre-leptotene/leptotene spermatocytes (B, C, H and I); white arrowhead points to zygotene spermatocytes (B, C, H and I); dark arrowhead points to pachytene spermatocytes (B, C, H and I); double arrow points to round spermatids (C and I); E indicates elongating spermatids (C and I); v-shaped arrowhead points to pertibular myoid cells; I indicates interstitial cell population where Leydig cells are found. Scale bar represents 50 μm for A–C and G–I, 50 μm for D and J, 80 μm for E–F and K–L and 12.5 μm for magnified images of individual cells.

remained unchanged through 91 dpp. The testicular content of activin A showed parallel changes until 25 dpp but thereafter showed a significant ($P<0.001$) increase to 0.03±0.002 g/testis at 91dpp.

Testicular FST concentrations (Fig. 5D) were significantly elevated at 3 dpp compared with 1 dpp ($P<0.01$), coinciding with the peak in activin levels, and continued to rise, peaking at 8 dpp (90.4±8.6 ng/g testis, $P<0.001$ compared with 10 dpp). Thereafter, the levels decreased ($P<0.001$) to a nadir at 25 dpp (28.2±0.6 ng/g testis) and remained unchanged until 91dpp. The total testicular content of FST levels showed a different profile, increasing continuously with increasing age to peak at 91 dpp (3.0±0.1 ng/testis).

Serum hormone concentrations

Serum activin A levels were the highest during the first 8 days of age (averaging 0.55±0.02 ng/ml) and decreased significantly ($P<0.001$) at 10 dpp continuing to decline in successive ages to reach a nadir at 45 dpp (0.05±0.002 ng/ml, $P<0.001$) (Fig. 6). Serum FST levels steadily increased from birth, peaking at 8 dpp (9.2±0.4 ng/ml, $P<0.05$ compared with 0 dpp) and remained at similar levels to 91 dpp.

Serum total inhibin levels showed a significant peak between 2 and 3 dpp (maximum of 5.5±0.1 ng/ml, $P<0.01$), with progressively lower values measured at 10 dpp ($P<0.05$) and at 45 and 91 dpp (minimum 1.0±0.2 ng/ml, $P<0.001$).

FSH and inhibin levels were negatively correlated throughout development ($r=-0.72$, $P<0.01$). During the first 10 days of neonatal life, FSH levels were at their lowest values, significantly declining between 2 and 4 dpp ($P<0.01$) and remaining low to 10 dpp (1.6±0.2 ng/ml). Subsequently, while inhibin levels declined, FSH levels rose between 10 and 16 dpp ($P<0.001$) with an additional significant increase measured at 25 dpp (11.0±0.5 ng/ml, $P<0.05$) and remaining elevated thereafter.

Discussion

This study provides the first assessment of the expression of the activins and inhibins, their binding protein, FST and FSH during testicular development in the normal

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Table 1 Summary of the intensity of *in situ* hybridisation signal for RNAs encoding *Inhba* and *Inhbb* genes and of immunohistochemsitry signal for INHA, INHBA, INHBB and INHBC proteins.

	mRNA				Protein			
	0 dpp	3 dpp	15 dpp	Adult	0 dpp	3 dpp	15 dpp	Adult
Gonocytes	*Inhba*− *Inhbb*−				INHA− INHBA+[#] INHBB+ INHBC+[N]			
Mitotic spermatogonia		*Inhba*+ *Inhbb*+	*Inhba*+ *Inhbb*+	*Inhba*++ *Inhbb*++		INHA− INHBA+ INHBB+ INHBC+[N]	INHA− INHBA+ INHBB+ INHBC−	INHA− INHBA− INHBB− INHBC−
Meiotic pre-leptotene, leptotene & zygotene spermatocytes			*Inhba*++ *Inhbb*++	*Inhba*++ *Inhbb*++			INHA− INHBA[N] INHBB+ INHBC+	INHA− INHBA[N] INHBB− INHBC+
Meiotic pachytene spermatocytes			*Inhba*+ *Inhbb*+	*Inhba*+ *Inhbb*+			INHA− INHBA+ INHBB+ INHBC−	INHA− INHBA± INHBB− INHBC−
Round spermatids				*Inhba*+ *Inhbb*+				INHA− INHBA− INHBB− INHBC+[N]
Elongating spermatids				*Inhba*− *Inhbb*−				INHA− INHBA± INHBB+ INHBC−
Sertoli cells	*Inhba*+ *Inhbb*+	*Inhba*+ *Inhbb*+	*Inhba*+ *Inhbb*+	*Inhba*+ *Inhbb*+	INHA+[#] INHBA+ INHBB+ INHBC+	INHA+[#] INHBA+ INHBB+ INHBC+	INHA+* INHBA+ INHBB+ INHBC+	INHA+* INHBA+ INHBB+ INHBC+[N]
Leydig cells	*Inhba*+ *Inhbb*+	*Inhba*+ *Inhbb*+	*Inhba*+ *Inhbb*+	*Inhba*+ *Inhbb*+	INHA+ INHBA+ INHBB+ INHBC+	INHA+ INHBA+ INHBB+ INHBC+	INHA+ INHBA+ INHBB+ INHBC+	INHA± INHBA+ INHBB+ INHBC+

+readily detectable signal; ++the signal is more intense than other positive cells; ± a faint signal; −no signal; [N]the signal is detected in the cell nucleus; [#]varying signal intensity across the cell population within tubule cross-sections; *signal intensity varies with the cycle of the seminiferous epithelium.

mouse. It provides baseline data that give insight into the roles of these proteins in normal testicular development and will assist future studies of these parameters in genetically modified or experimentally manipulated mice that exhibit perturbed testis function or altered spermatogenesis.

Activins and inhibins are involved in local actions that influence spermatogenic development and also participate in endocrine feedback regulation of the gonadotrophic hormone, FSH. Figure 7 illustrates the testicular protein levels of activin, inhibin and FST in relation to key spermatogenic events during mouse post-natal testis development. Here, inhibin levels are presented as amount per Sertoli cell, using previously published Sertoli cell numbers (Vergouwen *et al*. 1993), as it is widely understood that within the testis, inhibin is exclusively produced by the Sertoli cells (Steinberger 1979, Le Gac & de Kretser 1982). The changing concentrations and localisation of these proteins within the testis indicate precise temporal changes that point to a significant degree of regulation through mechanisms that are yet to be identified.

Much more is known concerning the changes in the testicular and circulating levels of the inhibins in other species through the measurements of inhibin A and B by the use of specific ELISAs. Unfortunately, the current ELISAs do not function for murine samples and hence we report total immunoactive inhibin measurements obtained using an assay that measures both inhibin A and B, and cross-reacts significantly with products of the α subunit (Robertson *et al*. 1988). Total testicular levels of inhibin in the mouse rise from 0 dpp to a peak at 4 dpp, with a subsequent plateau to day 16 that is followed by a subsequent decline; this parallels the pattern of changing inhibin B testis concentrations in the rat (Buzzard *et al*. 2004). The rise and plateau reflect a period of Sertoli cell proliferation in both species that culminates in the formation of a stable, non-mitotic Sertoli cell population. It is accepted that the total number of Sertoli cells achieved at this time determines

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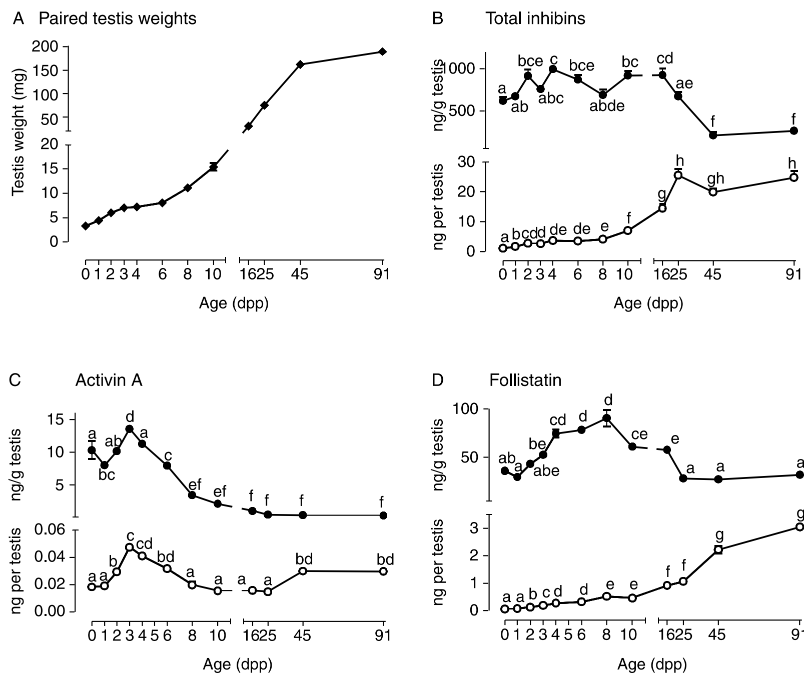

Figure 5 Paired testis weights and testicular levels of proteins in mouse post-natal development. (A) Paired testis weights and (B) testicular concentrations (ng/g testis; –●–) of total inhibins, (C) activin A and (D) follistatin proteins are shown. Total testicular production (ng/testis) for each protein is represented as ○. Data are shown as mean±S.E.M. of $n=6$ or more per group. Different letters at each point represent statistically significant data (refer to results section for P values).

the germ cell content and spermatogenic capacity of the adult testis, which in turn is reflected in the secreted levels of inhibin B.

In both the mouse and rat, there is a progressive rise in the testis inhibin content, stimulated by rising levels of FSH associated with the onset of sexual maturation. In the rat, this is also reflected in rising levels of inhibin B in serum (Sharpe *et al.* 1999, Buzzard *et al.* 2004), but surprisingly, in the mouse, the inhibin levels in the circulation do not rise to reflect the increased total testis inhibin content. The reason for this difference is unclear, as all other species such as sheep, sub-human primates and human show a parallel rise in serum inhibin and FSH levels associated with pubertal maturation. This is viewed as establishment of a new set point for the negative feedback relationship between inhibin and FSH in the adult mammal (Marson *et al.* 1991, Andersson *et al.* 1997, Kondo *et al.* 2000, Sanford *et al.* 2000, Crofton *et al.* 2002). Humans exhibit two distinct phases of Sertoli cell mitotic divisions, one during the first year of birth and then again at the onset of puberty, each associated with increased FSH stimulation (Andersson *et al.* 1997, Crofton *et al.* 2002, O'Connor & De Kretser 2004, Radicioni *et al.* 2005). In mice, however, our data show that FSH levels are the lowest during the period of Sertoli cell proliferation and increase after Sertoli cell maturation, with the negative relationship to inhibin established shortly after birth. This difference between humans and mice has been attributed to the measurable period of separation between post-natal and pubertal periods in humans, which does not exist in mice; spermatogenesis starts several years after birth in humans but begins within a few days after birth in mice. The FSH profile observed in the mice reflects its changing physiological role, from a mitogen acting on proliferating Sertoli cells during early post-natal development (Orth *et al.* 1988, Boitani *et al.* 1995, Meehan *et al.* 2000, Buzzard *et al.* 2003, Johnston *et al.* 2004, Meachem *et al.* 2005), to a stimulant on terminally differentiated Sertoli cells, ensuring the survival of germ cells and sperm production (Culler & Negro-Vilar 1988, Rivier *et al.* 1988, McLachlan *et al.* 1995, Meachem *et al.* 1998).

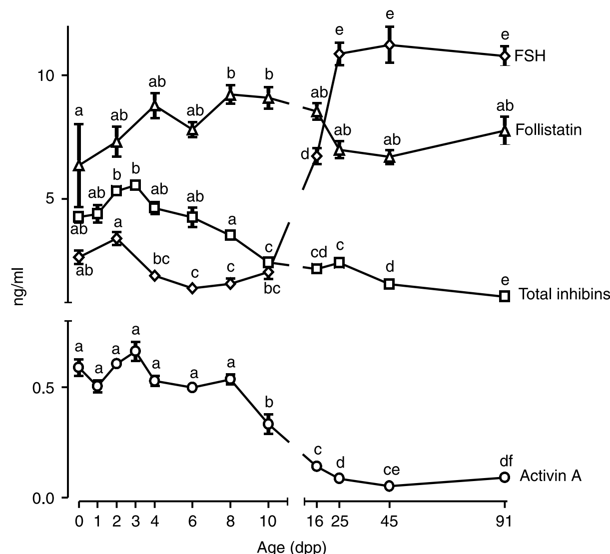

Figure 6 Levels of circulating hormones in mouse post-natal development. Serum hormone levels in mice are represented with respect to their age: (◇) FSH; (△) follistatin; (□) total inhibins; and (○) activin A (refer to Materials and Methods for numbers of mice). Data are shown as mean±S.E.M. of $n=6$ or more per group. Different letters on each line graph represent statistically significant data (refer to results section for P values).

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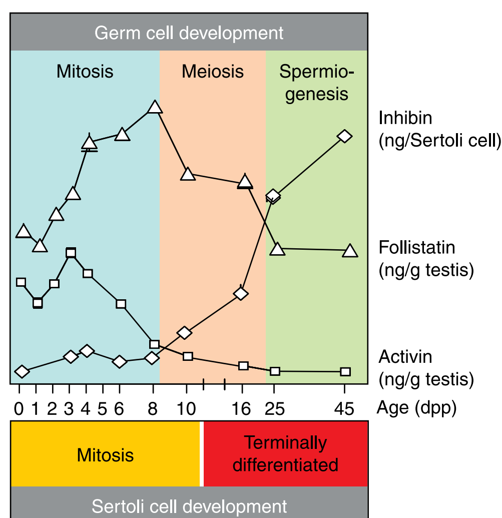

Figure 7 Modulation of testicular activin, inhibin and follistatin correspond with key spermatogenic events during mouse post-natal development. The activin and follistatin profiles expressed as ng/g testis are those presented in Fig. 5. Based on the assumption that inhibin is exclusively produced by Sertoli cells, inhibin values are presented as amount (ng) per Sertoli cell. Germ cells undergo three distinct developmental phases during spermatogenesis: mitosis, meiosis and spermiogenesis, the latter involving the morphological transformation of round spermatids to elongated mature spermatozoa. Sertoli cells are mitotically active during the phase of germ cell mitosis. They cease proliferation and enter terminal differentiation by around 11 dpp.

In accord with the results of studies in rats (Majdic *et al.* 1997) and humans (Anderson *et al.* 1998, Marchetti *et al.* 2003), the inhibin α subunit protein is detected primarily in Sertoli cells at all stages of post-natal development. Although this does not indicate the presence of dimeric inhibin, our observation of the β_B subunit mRNA and protein in mouse Sertoli cells suggests that they have the capacity to produce inhibin B and makes it highly likely that, in mice, the Sertoli cells are the major site of inhibin production. When Sertoli cells cease dividing, the inhibin concentration subsequently declined, evidently due to the dilution of Sertoli cell-derived products within the rapidly expanding germ cell population. On the other hand, total testicular production of inhibin significantly increased, indicating an increase in Sertoli cell activity, stimulated by high FSH levels.

Immunohistochemical analyses also revealed differential signal intensities for the inhibin α and β_B subunit proteins in Sertoli cells of the juvenile and adult mouse testes, with the highest signal intensity discerned at stages X–III of the adult. This differential expression is understood to reflect the spatial and temporal cycling of Sertoli cells. Inhibin B production in rat seminiferous tubules was shown to be the greatest at comparable stages (Okuma *et al.* 2006). This cyclic pattern of inhibin production is consistent with the fact that germ cells have a direct influence on inhibin production that is mediated via secreted factors (Pineau *et al.* 1990, Clifton *et al.* 2002). The physiological significance of this regulation across stages of the seminiferous epithelium may be an indication of the tight regulation of activin activity at specific stages and cell types, in concert with the presence of other activin inhibitors such as FST and the β_C subunit, to coordinate processes within the seminiferous epithelium. The regulation of activin activity by inhibin is also important during post-natal testis development as inhibin α null (*Inha*$^{-/-}$) mice develop visible stromal cell tumours at 4 weeks of age (Matzuk *et al.* 1992) mediated by the uninhibited actions of activin (Coerver *et al.* 1996, Li *et al.* 2007). In general, the modulation of inhibin expression during mouse spermatogenesis highlights its importance in maintaining testicular homeostasis.

In situ hybridisation and immunohistochemistry are only specific to the individual inhibin subunits being investigated and do not reveal the presence of dimeric proteins. However, these techniques identify the sites of cellular expression and hence possible sites of protein production. While the β_B subunit mRNA was detected in spermatogonia, spermatocytes and spermatids of developing and adult mouse testes, the encoded protein was only observed in spermatogonia and spermatocytes of the immature testis but not in the adult. As inhibin α subunit protein was only observed in Sertoli cells, it is likely that β_B subunit expression in germ cells reflects activin B production. It follows then that activin B may be important in the regulation of immature germ cell development but not in adult spermatogenesis, a prospect that correlates with the observation of lower β_B mRNA levels in the adult testis. Although male β_B null (*Inhbb*$^{-/-}$) mice exhibit normal fertility (Vassalli *et al.* 1994), a histological examination of their immature testes was not reported. It is possible that the β_A subunit compensates functionally for the absence of the β_B subunit in this model, explaining its normal fertility phenotype. Currently, there is no assay available to measure activin B levels, and so the physiological relevance of activin B remains to be determined. Our observation of β_B subunit in adult Sertoli and Leydig cells agrees with published data on adult rat testes (Majdic *et al.* 1997, Buzzard *et al.* 2004) and human testes (Anderson *et al.* 1998). Only one report (Marchetti *et al.* 2003) observed the β_B subunit protein in human pachytene spermatocytes and round spermatids but not in Sertoli cells. Although these discrepancies may be attributed to differences in tissue fixation and antigen retrieval, they may also reflect differential synthesis of inhibin and activin between the species.

Serum levels of activin A observed in this present study of mice mimicked the profile reported previously for rats (Buzzard *et al.* 2004), but its physiological significance remains unknown. While the source of circulating inhibin is predominantly the testis, the source of activin is ubiquitous, being synthesised by a large number of

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tissues (Meunier *et al.* 1988*b*). Furthermore, activin is widely viewed as a paracrine, rather than endocrine, regulator of cellular development (Welt & Crowley 1998). Hence, the following discussion will focus on the correlation between testicular activin levels and its cellular localisation with physiological events.

In vitro studies of rat testes have shown that activin plays a key role in Sertoli cell proliferation (de Winter *et al.* 1993, Boitani *et al.* 1995, Brown *et al.* 2000, Fragale *et al.* 2001, Wreford *et al.* 2001, Buzzard *et al.* 2003). We observed the β_A subunit mRNA and protein in mouse Sertoli cells and peritubular myoid cells throughout testis development. While the effect of activin on peritubular myoid cell growth has not been reported, peritubular myoid cells have been shown to secrete activin A *in vitro* and act in a paracrine fashion on Sertoli cells, inducing their production of inhibin and transferrin (de Winter *et al.* 1994). These data, along with our observations of elevated activin levels during the period of Sertoli cell proliferation, suggest that activin has both autocrine and paracrine actions on Sertoli cells during mouse testis development. This is supported by the finding that mice deficient in the activin type II receptor (Matzuk *et al.* 1995*b*) and mice with reduced activin bioactivity (Brown *et al.* 2000) have smaller testis size, indicative of reduced Sertoli cell numbers. Similarly, over-expression of the activin antagonists, FST and FST-like 3 (FSTL3), results in reduced testicular size in mice (Guo *et al.* 1998, Xia *et al.* 2004). Testes from both of these transgenic lines contained degenerating tubules devoid of germ cells and produced significantly less sperm, suggesting that germ cell development is compromised when activin activity is reduced.

Leydig cells also produce inhibin and activin subunits. We report here that both β_A and β_B subunit mRNAs and proteins are present in Leydig cells during post-natal testis development. Inhibin α subunit protein was also observed in Leydig cells, with stronger staining at 0 dpp than at older ages. Majdic *et al.* (1997) also reported expression of inhibin α and β_B subunit proteins in Leydig cells of rat testes, with decreasing signal intensity from birth to adulthood. Furthermore, adult rat Leydig cells have been reported to secrete bioactive and immunoreactive inhibin *in vitro* in response to LH (Drummond *et al.* 1989, Risbridger *et al.* 1989), and normal men administered with human chorionic gonadotrophin (hCG) had in increase in blood levels of pro-αC (free α subunit) but not inhibin B (Kinniburgh & Anderson 2001). These data imply that Leydig cells produce inhibin, although it is not secreted into circulation as inhibin B. Other lines of evidence indicate that Leydig cell inhibin plays autocrine and/or paracrine functions. In cultures of Leydig cells isolated from 21 dpp rats, inhibin was found to stimulate testosterone production and antagonise the testosterone-suppressive effects of activin A (Hsueh *et al.* 1987). Subsequent studies on adult Leydig cells, however, did not identify testosterone-stimulating actions of inhibin. While inhibin alone did not influence testosterone production, it did effectively block activin suppression of testosterone production by adult Leydig cells (Lin *et al.* 1989, Risbridger *et al.* 1989). These data suggest that some functions of inhibin may be age specific or they may relate in cell culture studies to the levels of endogenous production, which we have highlighted are dynamic throughout testicular development.

Because androgens are important regulators of Leydig cell development and function, it may be hypothesised that activin and inhibin influence Leydig cell development through the steroidogenic pathway. However, while testes from mice with reduced activin bioactivity, including ActRII knockout mice (Matzuk *et al.* 1995*b*), FST transgenic mice (Guo *et al.* 1998) and FSTL3 transgenic mice (Xia *et al.* 2004), have increased Leydig cells numbers, their LH and testosterone levels remain similar to those in wild-type animals. Only one FSTL3 transgenic mouse line showed elevated testosterone levels at 21 dpp and reduced testosterone levels at adulthood (Xia *et al.* 2004). Hence, activin may act as a negative regulator of Leydig cell proliferation, independent of its effects on androgen production. Inhibin also negatively influences Leydig cell proliferation in synergy with Mullerian Inhibiting Substance (MIS), as mice deficient in both inhibin α and MIS display an earlier and more aggressive onset of Leydig cell neoplasia than do their respective homozygote knockouts (Matzuk *et al.* 1995*a*). In summary, our observation of the activin/inhibin subunits in Leydig cells is in agreement with current data on their autocrine and paracrine regulation of Leydig cell function and proliferation.

We observed the localisation of β_A mRNA and protein in spermatogonia and spermatocytes of immature and adult mouse testes, but the ability of activin to regulate these cells depends on the expression of the activin receptors. The activin type II receptor mRNA has been shown to be expressed by Sertoli and germ cells (spermatogonia to round spermatids) of immature and adult rat testes (de Winter *et al.* 1992, Fragale *et al.* 2001), and one report shows both type IIB and type I activin receptor mRNAs are synthesised by immature murine spermatogonia and Sertoli cells (Puglisi *et al.* 2004), suggesting that activin may mediate both autocrine and paracrine regulation of both cell types.

The frequent sampling during the first week *post partum* which is a feature of this study enabled the identification of significant changes in testicular activin A concentrations, suggesting a tight modulation of activin signalling during this period. First, activin A concentrations significantly declined between 0 and 1 dpp coinciding with the start of gonocyte proliferation and comparable with the decline observed in the rat between 0 and 3 dpp (Buzzard *et al.* 2004). Subsequently, activin levels rose to peak at 3 dpp, after which the first spermatogonial stem cells appear (de Rooij 1998, McLean *et al.* 2003, Yoshida *et al.* 2004). These observations suggest a dual role of activin regulation

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on gonocyte development, where a reduction in local activin concentration is required for gonocytes to exit from their state of G1 arrest, while subsequent elevation in activin levels suggests a role in promoting gonocyte proliferation. The role of activin as an inducer of mitotic arrest in gonocytes is evident in the elevated gonocyte numbers in testes of newborn mice lacking the β_A subunit (*Inhba*$^{-/-}$; Loveland *et al.* 2005). Subsequently, the role of activin as a mitogen is reflected in a study of 3-day-old rat testis fragment cultures, which showed that increased activin levels induced the proliferation of gonocytes (Meehan *et al.* 2000). In the present study, the observation of the β_A subunit protein in a subset of gonocytes and a more general localisation of the β_B subunit in gonocytes supports the regulatory action of activins on these cells, but of course it may also influence the Sertoli cell population in which they are embedded.

Activin levels decrease after the differentiation of gonocytes into spermatogonia concordant with a rise of FST and inhibin testicular concentrations, both of which can inhibit the biological actions of both activin A and B. An increase of FST protein within spermatogonia and Sertoli cells of the immature rat testis has been previously described (Meehan *et al.* 2000). The rapid downregulation of activin activity would be envisioned to limit its mitogenic actions on gonocytes and Sertoli cells. This suggests that there is a need to inhibit activin bioactivity to enable gonocytes to differentiate into spermatogonia (Meehan *et al.* 2000) and to allow proliferation of spermatogonia, as activin has been shown to suppress the proliferative actions of FSH specifically on differentiating type A spermatogonia (Boitani *et al.* 1995). Furthermore, over-expression of the β_A subunit in juvenile mouse testes caused degeneration of spermatocytes and resulted in sterility (Tanimoto *et al.* 1999). Considered together with our reported observation of elevated β_C subunit mRNAs in the mouse testis at the time of meiosis, the available data indicate that reduced activin bioactivity is required for spermatogonial proliferation and differentiation.

The expression of the β_A subunit in the nucleus of spermatocytes at 15 dpp and adult age is in agreement with a previous report (Blauer *et al.* 1999), but its role at this stage is unknown. Similarly, the β_B subunit detected in primary spermatocytes is noted and may be related to the influence of activin action on the morphology of mitochondria in these cells and their changing role in metabolism (Meinhardt *et al.* 2000). The emerging picture of regulated production and action of activin throughout testis development is in accord with its function as a key governor of testis growth.

While the role of the β_C subunit in testis development is uncertain at this time, it has been reported to function as a dominant negative regulator of activin A activity by sequestering the β_A subunit to produce the activin AC heterodimer (Mellor *et al.* 2003), and hence local activin A bioavailability may be limited by co-expression of both β_C and β_A subunits. We have shown that both are present in Sertoli and Leydig cells in immature and adult testes, as well as in spermatocytes and elongating spermatids of the adult mouse. However, while the β_A subunit protein was evident in the nucleus of primary spermatocytes, the β_C subunit protein, which also encodes a nuclear localisation signal (Lau *et al.* 1996), was selectively observed at a later developmental stage in the nucleus of round spermatids. It is therefore possible that the β_C subunit performs additional roles, independent of antagonising β_A signalling activity.

In summary, this study has documented that the levels of activins, inhibins, FST and FSH are highly modulated during mouse testis development, and changes in mRNA and protein levels correlate with shifts in Sertoli cell maturation and phases of germ cell development. In addition, the inhibin/activin subunit mRNAs and proteins exhibit discrete and overlapping cellular localisation patterns during the course of testis development and in the adult testis. The rapid changes in levels of activin A and its negative regulators during the onset of the first wave of spermatogenesis highlights the importance of understanding the regulation and control of activin signalling. Our data show this may occur through the dynamic synthesis of FST, inhibin α- and inhibin/activin β_C-subunit proteins. This is the first comprehensive assessment of cellular production of activins, inhibin, FST and FSH levels in the developing mouse testis. These data offer a rigorous platform for further studies of the roles of these proteins during normal and perturbed testis growth throughout the first wave of spermatogenesis.

Materials and Methods

Animals and tissue collection

Testes used for RNA and protein isolation and serum samples were obtained from C57BL/6xCBA F1 male mice aged 0, 1, 2, 3, 4, 6, 8, 10, 16, 26, 42 and 91 days *post partum* (dpp). These mice were housed at Monash University Central Animal Services (MAS) under a 12-h light cycle. Ovarian and hepatic tissue used for RNA isolation (providing controls for real-time PCR studies) were obtained from Swiss mice housed at MAS. Testes used for histological analysis were obtained from Swiss mice housed at the University of Newcastle Central Animal Facility. The day of birth was noted as 0 dpp. Mice up to the age of 16 days were decapitated and blood immediately collected using a Microvette CB 300 capillary tube (Sarstedt, Germany). Older mice were killed by CO_2 asphyxiation and blood collected via cardiac puncture. Blood samples were centrifuged at 7500 ***g*** for 20 mins, and serum was collected and stored at −20 °C.

For RNA isolation and testes protein homogenates, testes were decapsulated, weighed, snap frozen and stored at −80 °C until use. For *in situ* hybridisation, testes were immediately placed in Bouin's fixative, and after 5 h they were washed several times in 70% ethanol and processed as described below. All investigations

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conformed to the NHMRC/CSIRO/AAC Code of Practice for the Care and Use of Animals for Experimental Purposes and were approved by the Monash University Standing Committee on Ethics in Animal Experimentation and by the University of Newcastle Animal Care and Ethics Committee.

Quantitative mRNA analysis

Total RNA from mouse testes was extracted using TRIzol reagent (Invitrogen) and treated with DNA-free (Ambion, Austin, TX, USA) according to the manufacturer's specifications. Three independent tissue samples were used to prepare RNA samples for each time point. Each 0 dpp RNA sample was made from a pool of eight testes, each 4 dpp RNA sample from a pool of six testes, 6–16 dpp from a pool of four testes, and 26 and 42 dpp from a pool of three testes. Two micrograms of DNA-free RNA were used to generate cDNA using Superscript III reverse transcriptase (RT) (Life - Technologies) with oligo-dT primers, according to the enzyme manufacturer's guidelines. Negative control RT samples lacking Superscript III (−RT) were included for each sample. PCR samples were prepared using Power SYBR-Green PCR master mix (Applied Biosystems, Foster City, CA, USA) and the gene-specific primers listed in Table 2. PCR was performed in the Applied Biosystems 7900HT Analyzer (Applied Biosystems) using the following cycling conditions: denaturation at 95 °C for 10 min; amplification for 40 cycles of 95 °C for 15 s, 62 °C for 30 s and 72 °C for 30 s. Each sample was analysed in triplicate and quantification performed using the Pfaffl equation (Pfaffl 2001) relative to levels of actin (*Actb*) mRNA. The values were then normalised to the value at 0 dpp, which was arbitrarily set to 1. Water and −RT samples were included as negative controls. PCR products were verified by sequencing.

In situ *hybridisation*

In situ hybridisation using digoxygenin (DIG)-labelled cRNAs was used to localise mRNAs encoding *Inhba* and *Inhbb* in Swiss mouse testis sections as previously described (Meehan *et al*. 2000). Briefly, Bouin's fixed testes were embedded in paraffin and cut into 4 μm sections. Using the primers in Table 2, probes were prepared from cDNA templates and their specificity verified using sequencing and Northern blot analyses. Sense and anti-sense DIG-labelled cRNAs for each target sequence were made from purified PCR products (QIAquick PCR Purification Kit; QIAGEN) using RNA polymerises T7 and SP6 (Promega) and the DIG-labelling kit (Roche) according to the manufacturer's instructions.

Hybridisation was performed overnight at the following probe concentration and temperature for each target sequence: 1 μg/ml and 50 °C for *Inhba* and 1 μg/ml and 55 °C for *Inhbb*. The probes were detected with anti-DIG antibody (Roche) and visualised using 5-Bromo-4-chloro-3-indolyl phosphate/Nitroblue tetrazolium (BCIP/NBT; Astral Scientific, Sydney, Australia). The sections were counterstained with Harris haematoxylin (Sigma Chemical Co.) and mounted in GVA aqueous mounting solution (Invitrogen). In every experiment, on every sample, the sense cRNA was included in parallel with the anti-sense cRNA as a negative control.

For these studies and also for immunohistochemisty, the qualitative assessment of cellular localisation was performed on at least seven different tubule cross-sections from three different animals for ages 0 and 3 dpp, and at least six different tubule cross-sections of each stage of the seminiferous epithelium from at least two different animals for ages 15 dpp and adult.

Immunohistochemistry

Immunolocalisation, using antibodies to INHBA (#153+154 anti-cyclic activin β_A (81–113)-NH_2 rabbit polyclonal) and INHBB (#197 anti-cyclic activin β_B (80–112)-NH_2 rabbit polyclonal; gifts from Wylie Vale at the SALK Institute; Meunier *et al*. 1988*a*, Roberts *et al*. 1989, Vaughan *et al*. 1989, Bilezikjian *et al*. 1993), INHBC (anti-activin β_C Clone-1; Mellor *et al*. 2000) and INHA (anti-inhibin α subunit R1; Serotec, Oxford, UK) were detected using the DAKO Autostainer Universal Staining System (DAKO, Glostrup, Denmark) as previously described (Gold *et al*. 2004). Microwave antigen retrieval was carried out in 0.01 M citrate buffer (pH 6.0) for INHBA, INHBB and INHA and 0.01 M glycine buffer (pH 4.5) for INBBC. Sections were treated with CAS Blocking Reagent (DAKO), antibodies detected with avidin–biotin complex (ABC) and a colour reaction product developed following addition of 3,3′-diaminobenzidine tetrahydrochloride (DAB; DAKO). Negative controls were immunoglobulins (IgG) matched to the primary antibody, rabbit IgG (DAKO) for INHBA and INHBB, IgG2a (DAKO) for INHA and IgG1 (DAKO) for INHBC.

Serum and testicular extracts

To have sufficient serum for use in the hormone assays, equal volumes of serum from several animals were pooled for the 0–16 dpp groups as follows. The numbers of mice sampled per pool were five to eight mice for the 0 dpp group, two to three mice for the 1, 4, 6, 8 and 16 dpp groups and four to five mice for the day 2 group. For 25, 43 and 91 dpp groups, six to

Table 2 Primers used in real-time PCR.

Gene name	Common name	Accession no.	Forward primer	Reverse primer
Inha	Inhibin α	NM_010564	5′-ATGCACAGGACCTCTGAACC-3′	5′-GGATGGCCGGAATACATAAG-3′
Inhba	Inhibin/activin β_A	NM_008380	5′-GGAGAACGGGTATGTGGAGA-3′	5′-TGGTCCTGGTTCTGTTAGCC-3′
Inhbb	Inhibin/activin β_B	NM_008381	5′-CCTGAGTGAATGCACACCAC-3′	5′-CGAGTCCAGTTTCGCCTAGT-3′
Inhbc	Inhibin/activin β_C	NM_010565	5′-GACTCCAACCACAGTAGTGAAC-3′	5′-CACTGGCCGACTGAGTATGG-3′
Fst	Follistatin	NM_008046	5′-AAAACCTACCGCAACGAATG-3′	5′-TTCAGAAGAGGAGGGCTCTG-3′
Actb	Actin	NM_007393	5′-AGGCTGTGCTGTCCCTGTAT-3′	5′-AAGGAAGGCTGGAAAAGAGC-3′

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eight individual animals were used. For each age group, six to eight pooled samples were generated and analysed.

To generate testicular homogenates, paired testes from the same mice used for the serum pools were decapsulated and homogenised in PBS on ice using a hand-held tissue homogeniser. The mass of testis per ml of PBS for homogenates was 60 mg tissue per ml for 0–16 dpp pools and 200 mg/ml for 25–91 dpp groups. Homogenates were spun for 20 min at 10 000 ***g***, and the supernatants were stored at −80 °C. Final protein concentrations of the samples were matched to the protein content of the assay diluents.

Hormone assays

Serum FSH concentrations were determined using RIA reagents kindly provided by Dr A Parlow, NIDDK (Bethesda, MD, USA). The iodination preparation and antiserum used were rFSH I-8 and anti-rFSH-S-11 respectively and the results are expressed in terms of NIDDK mFSH-RP-1. Goat anti-rabbit IgG (GAR#12; Monash Institute of Medical Research, Monash University, Melbourne, Australia) was used as second antibody. All measurements were performed on 20 μl duplicates of serum. The lowest limit of detection was 1.14 ng/ml, the average within-assay coefficient of variation (CV) was 7.4%, and the inter-assay CV was 3.0% ($n=3$ assays) calculated using a pool of normal mouse serum used in each assay.

The available ELISA assays for measuring inhibin A (Groome *et al.* 1994) and inhibin B (Groome *et al.* 1996) proteins do not detect these proteins in mice. Hence, inhibin levels were measured using a RIA with a rabbit antiserum (#1989) directed to the α subunit and as such measures both inhibin A and B proteins and free inhibin α subunit (pro-α-C; Robertson *et al.* 1988). Results are expressed in terms of 31 kDa hr-inhibin, and iodinated hr-inhibin was used as tracer. Goat anti-rabbit IgG (GAR#12) was used as second antibody. The average within-assay CV was 9.1% and the inter-assay CV was 5.0% ($n=4$ assays). The lowest limit of detection was 0.22 ng/ml.

FST concentrations were measured using a discontinuous RIA as described previously (O'Connor *et al.* 1999), which detects total FST using a dissociating reagent that dissociates the activin–FST complex. The samples were measured in a total of five assays, the average intra-assay CV was 10.1%, the inter-assay CV was 6.2% and the limit of detection was 0.99 ng/ml.

Activin A was measured using a specific ELISA (Knight *et al.* 1996) which exhibits minimal cross-reactivity with inhibin A (0.5%), and no significant cross-reactivity to other related proteins, according to the manufacturer's instructions (Oxford Bio-Innovations, Oxfordshire, UK) with some minor modifications as described previously (O'Connor *et al.* 1999). Protein concentrations in the serum and testis homogenates were matched to the BSA content in the assay diluent. For the serum assays, the average intra-plate CV was 5.4%, the inter-plate CV was 7.0% ($n=4$ plates) and the detection limit was 0.01 ng/ml. For the testis homogenate assays, the average intra-plate CV was 7.6%, the inter-plate CV was 9.7% ($n=3$ plates) and the detection limit was 0.01 ng/ml. A mouse serum pool and a testis homogenate pool were each diluted in a dose-dependent manner, which was parallel to the standard curve in each assay (data not shown).

Statistical analysis

Data are represented as mean±S.E.M. All data were analysed using one-way ANOVA combined with Tukey's post-test for comparison between each age. Serum data were log transformed before ANOVA analysis. Data from groups with different letters were significantly different ($P<0.05$). Correlation between inhibin and FSH was analysed using Pearson's test.

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