Differential expression of TGFBR3 (betaglycan) in mouse ovary and testis during gonadogenesis

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(Received 8 October 2007; revised 13 November 2007; accepted 27 November 2007)

Abstract
TGFBR3 is an accessory receptor that binds to and modulates the activities of both transforming growth factor-beta (TGFβ) and inhibin, two members of the TGFβ superfamily of growth factors that regulate many aspects of reproductive biology. Tgfbr3 is known to be expressed in adult testis and ovary, but little is known about this receptor during gonadogenesis. Herein, we describe Tgfbr3 expression in the male and female fetal and neonatal murine gonad. Real-time PCR analysis revealed that Tgfbr3 mRNA was expressed at higher levels in the developing testis compared to ovary. TGFBR3 was expressed within the fetal testis interstitium, predominantly by Leydig cells, but expression shifted inside the seminiferous cords at birth. In contrast, TGFBR3 was detected in both the somatic and germ cell lineages in the fetal and neonatal ovary. This differential expression pattern suggests divergent roles for this TGFBR3 in developing testis and ovary.

Keywords: Betaglycan, TGFβ type III receptor, inhibin, Leydig cell, gonadogenesis

Introduction
Male and female gonad differentiation in the mouse involves the coordinated behaviors of multiple cell types over the fetal and neonatal developmental periods. Beginning at 10.5 days post-coitum (dpc), the genital ridge is colonized by primordial germ cells (PGCs). Proliferation of PGCs and somatic cells in the genital ridge gives rise to the bipotential gonad, which first becomes discernible from the underlying mesonephros around 11.5 dpc. The testis and ovary become morphologically distinct between 12.0 and 12.5 dpc, when male-specific structures, the seminiferous cords and coelomic vessel, emerge within the testis under the control of the Y-chromosome Sry gene (Brennan et al. 2002). The seminiferous cords arise from pre-Sertoli cells, the key support cells for the germ cells, migrate into the gonad from the coelomic epithelium and aggregate around the germ cells (Karl and Capel 1998). Peritubular cells migrate from the adjacent mesonephros and cooperate with the Sertoli cells to establish a basement membrane (Skinner et al. 1985; Nishino et al. 2001). This basement membrane delineates the cords, composed of Sertoli, peritubular and germ cells, from the interstitium, which contains stromal and fetal Leydig cells. During fetal development, the testis grows rapidly due to the proliferation...
of the somatic cell populations. In contrast, the germ cells (also termed gonocytes) begin to undergo mitotic arrest around 13.5 dpc and do not resume their proliferation until after birth, when they migrate to the cord basement membrane and begin differentiating. Precise regulation of these morphological and cellular events during testis development is required for the proper formation of the adult testis. In particular, the establishment of the populations of both the gonocytes and their essential supporting cells, the Sertoli cells, during fetal and neonatal development determines adult male reproductive function (for review, see Loveland and Robertson 2005).

Similarly, adult female reproductive capacity is thought to be determined during ovarian development when the populations of germ cells (or oogonia) and key somatic cells (granulosa and theca cells) are established. Between 10.5 and 13.5 dpc, both oogonia and ovarian somatic cells undergo proliferation. Around 13.5 dpc, the oogonia begin meiosis and differentiate into oocytes (Bullejos and Koopman 2004). The fetal oocytes associate with each other and with pre-granulosa cells, forming loosely-structured ovigerous cords (Odor and Blandau 1969). The clusters of oocytes are interconnected by intercellular cytoplasmic bridges and are considered “germ cell cysts”. During the early neonatal period, the germ cell cysts break down into primordial follicles, as the pre-granulosa cells grow between the oocytes and separate them into discrete follicular units (Pepling and Spradling 2001). This process of neo-folliculogenesis occurs extensively in the neonatal period in rodents, establishing a pool of healthy primordial follicles, which will serve as the main follicular reserve throughout life (Bristol-Gould et al. 2006; Kerr et al. 2006).

Specific growth factors have been implicated in the regulation of morphological events within the developing testis and ovary. The transforming growth factor-beta (TGFβ) superfamily is a multifunctional group of growth factors which includes the TGFβs, activins, inhibins, growth and differentiation factors and bone morphogenetic proteins (BMPs). Several members of this superfamily participate in the autocrine and paracrine regulation of many cellular events underlying gonad development and function, including cellular proliferation, differentiation, adhesion, migration and extracellular matrix production. The diverse activities of TGFβ superfamily members are mediated by pairs of serine/threonine kinase receptors which form heteromeric complexes in which the type II receptor is necessary for binding ligand and the type I receptor is necessary for signal transduction. Signal transduction is mediated by the intracellular family of molecules, the Mothers against decapentaplegic-homologs (SMADs) (for review, see Shi and Massague 2003). The TGFβs (TGFβ1–3), activins and inhibins, affect the proliferation and death of germ and somatic cells of both ovary (Chuva de Sousa Lopes et al. 2005; Mendez et al. 2006) and testis (Meehan et al. 2000; Konrad et al. 2006) during development. The TGFβs also regulate the migration and differentiation of multiple cell types in fetal testis culture, which suggests that these factors may influence the development of fetal testis morphology (Skinner and Moses 1989; Cupp et al. 1999; Konrad et al. 2000). Specific roles for these growth factors in gonadogenesis in vivo have not been reported but are suggested by the reproductive phenotypes reported for Tgfb1, Tgfb2, Smad3, activin type II receptor (Actr2), inhibin-α (Inhba), and Inhbb null mice (Matzuk et al. 1992; Sanford et al. 1997; Tomic et al. 2004; Ma et al. 2005; Ingman et al. 2006; Yao et al. 2006; Ingman and Robertson 2007) and for mice harboring a conditional ovary-specific knockout of Smad4 (Pangas et al. 2006).

In addition to the signalling receptors, binding proteins which modulate TGFβ superfamily function are also present on many cell types. Of these, the TGFβ type III receptor (TGFBR3), also known as betaglycan, is the most abundant on many cell types (Segarini et al. 1989). TGFBR3 is a membrane-bound proteoglycan which can bind TGFβs and inhibins with high affinity and act as a non-signalling accessory receptor for these growth factors (Lopez-Casillas et al. 1993; Lewis et al. 2000; Wiater and Vale 2003). The presence of TGFBR3 on the cell surface increases the binding of the TGFβs to their type II receptors, an effect that is most pronounced for TGFβ2, which binds poorly to the TGFβ type II receptor in the absence of TGFBR3 (Lopez-Casillas et al. 1993). TGFBR3 also significantly increases the function of inhibin as an antagonist of activin and the BMPs (Lewis et al. 2000; Wiater and Vale 2003). The role of TGFBR3 as a TGFβ and inhibin co-receptor appears essential in certain organs during embryonic development, as Tgfb3 null mice generally die during late gestation of heart and liver defects (Stenvers et al. 2003). In the adult, Tgfb3 is widely expressed in many organs, including the pituitary (MacConell et al. 2002; Chapman and Woodruff 2003), ovary (Lewis et al. 2000; Drummond et al. 2002; MacConell et al. 2002; Chapman and Woodruff 2003; Liu et al. 2003), and testis (Lewis et al. 2000). These data suggest that TGFBR3 modulates the extragonadal and intragonadal actions of its ligands in the adult reproductive system, a conclusion which is supported by a recent report linking mutations in the human Tgfbr3 gene to premature ovarian failure (Dixit et al. 2006). In addition, the few Tgfb3 null male and female mice which survive to sexual maturity exhibited poor fertility (Stenvers et al. 2003), suggesting that TGFBR3 is essential to reproductive processes in both sexes. However, these studies examined the effects of germline mutations in the Tgfb3 gene on adult reproductive capacity. Therefore, it is currently
unclear in which organ and at which stage of development that Tgfbr3 is essential for normal reproductive function. To gain a better understanding of this receptor in reproductive biology, we characterized the expression patterns of the Tgfbr3 gene in murine ovary and testis at different stages of fetal and postnatal development. Although limited studies have been conducted previously in developing rat (Le Magueresse-Battistoni et al. 1995; Drummond et al. 2002) and human gonad (Anderson et al. 2002), this is the first study to examine the mRNA and protein expression patterns of this receptor throughout gonadal development in the mouse, from 12.0 dpc until 15 days post partum (dpp).

**Materials and methods**

**Tissue collection and processing**

Mouse embryos from timed matings of CD1 or C57B6 X sv129 female mice were obtained from pregnant dams between 12.0 and 16.5 dpc in half-day increments, with noon on the day of vaginal plugging designated as 0.5 dpc. To validate the TGFBR3 antibody, wildtype (WT) and knockout littermates were obtained from a Tgfbr3 null mouse colony and genotyped as previously described (Stenvers et al. 2003). Embryos were sexed by morphology and PCR (McCline and Sinclair 2001). Neonatal and prepubertal mice at 0 (day of birth), 2, 4, 5, 8, 10, 12 and 15 dpp were examined. In addition, the 30 dpp ovary was dissected away from mesonephroi and frozen at −80°C until RNA was extracted. For histology, urogenital systems were isolated from the embryos and neonates and were fixed in 4% paraformaldehyde, as detailed below. All animal handling and experimental procedures were approved by the Monash Medical Centre Animal Ethics Committee.

**Real-time polymerase chain reaction (PCR) analysis**

Total RNA was extracted from isolated gonads dissected free from the mesonephroi of fetal and neonatal mice using the RNeasy kit (Qiagen, Australia), as recommended by the manufacturer’s protocol. Contaminating DNA was removed from the RNA preparations using the DNA-free™ kit (Ambion, TX, USA). RNA was quantified using the ND-1000 Nanodrop spectrophotometer (NanoDrop technologies Inc., Wilmington, DE, USA). Complementary DNA was synthesized using Superscript™ III Reverse Transcriptase (Life Technologies, Grand Island, NY) using random hexamer and oligo d(T)20 primers, as previously described (Sarraj et al. 2005).

PCR samples were prepared to a final volume of 10 μl using the Applied Biosystems ABI SYBR mix (Scoresby, Victoria, Australia). PCR was performed using the Applied Biosystems ABI 7900 HT Fast real-time machine. Cycling conditions comprised of a 10 min initial denaturation step at 95°C, then 40 cycles of denaturing (95°C for 15 s), annealing (55°C for 30 s) and elongation (72°C for 30 s). The Tgfbr3 primers used for RT-PCR were Tgfbr3 forward (5'-CCTCCTCAGATTTCCA-3') and Tgfbr reverse (5'- CCCCCATCAACGCTCTGAG -3'). The primers amplified a fragment spanning an intron-exon junction of the mouse Tgfbr3 gene (AF039601), ensuring that the amplified products were not genomic in origin. 18S primers (NR_003278.1) 18S forward (5'- GAAACCCTGTTGAACCCATT-3') and 18S reverse (5'- CATTCATCGGTAGTAGCG -3') were taken from Schmittgen and Zakrajsek (2000). The PCR products were verified by cloning and sequencing. Three different sets of cDNA pools were used to perform replicates each comprising of seven to ten pairs of gonads from each sex. PCR reactions were performed in triplicate with negative controls, where water was used in place of the reverse-transcribed template, included for each primer pair to exclude PCR amplification of contaminating DNA. Tgfbr3 mRNA levels were normalized to that of 18S as previously described in detail (Sarraj et al. 2007). Analysis was performed using the ΔΔCT method, and statistical analysis was performed using one way Analysis of variance (ANOVA).

**Immunofluorescence**

Embryonic gonad tissues (12.0–14.5 dpc) were fixed in 4% paraformaldehyde at 4°C for 10 min, incubated in 20% sucrose overnight at 4°C, and embedded in an OCT-sucrose medium for frozen sectioning (8 μm). For fetal ages over 14.5 dpc and postnatal ages, 4% paraformaldehyde-fixed, paraffin-embedded tissues were used, and an antigen retrieval step was added, utilizing a commercially available antigen-unmasking reagent according to the manufacturer’s instructions (DAKO). Experiments were performed on at least three mice of each sex and age. For immunofluorescence, frozen sections were air dried at room temperature briefly then treated with 1% Triton-X in phosphate buffered saline (PBS) for 10 min at room temperature and rinsed with PBS. Sections were blocked with 5% donkey serum in PBS, and incubated overnight at 4°C with antibodies against TGFBR3 raised in goat (R&D Systems Cat# AF-242-PB; 1:50 dilution). Sections were then washed with PBS then incubated with a donkey anti-goat Alexa Fluor 488-conjugated secondary antibody for detection (Molecular Probes; donkey anti-goat; 1:200). Cell nuclei were visualized with either 4',6-diamidino-2-phenylindole-dihydrochloride (DAPI; Roche) or TO-PRO (Invitrogen). Slides were mounted in Fluorosave Mounting Medium (Calbiochem). Images were
captured on an Olympus FV300/IX81 confocal or BX60 upright fluorescent microscope. The TGFBR3 antibody was validated by performing assays in parallel on WT and knockout littermates from the Tgfbr3 gene knockout mouse line. Additional negative controls included incubating WT sections with the primary or secondary antibody alone or with goat IgG. Assessments of the relative levels of immunostaining were made on sections processed within the same assay and imaged under the same conditions. For co-localization experiments with TGFBR3 antibody, an antibody raised in rabbit against CYP11A1/P450-Side chain cleavage (Scc) enzyme (Chemicon Cat# AB1294) was used at a dilution of 1:400. Mouse MVH Homologue (MVH) antibody (Abcam Cat#ab13840) was raised in rabbit and used at a dilution of 1:1000. CYP11A1/P45-Scc and MVH signals were detected using a donkey anti rabbit Cy3 secondary antibody (Chemicon cat#AP182C, 1:200). All antibodies were diluted in 1% bovine serum albumin in PBS prior to use.

Results

**Tgfbr3 mRNA and protein expression during testis morphogenesis**

During fetal development, similar levels of Tgfbr3 expression were measured in gonads of both sexes between 12.5 and 14.5 dpc. Expression significantly increased in the testis during late gestation and at birth, with the newborn testis exhibiting a 3-fold increase over the levels observed in 12.5 dpc testes (Figure 1). Tgfbr3 expression in the ovary differed from the testis in that levels did not significantly change between 12.5 and 16.5 dpc. The level of expression in ovary was also significantly less than that in testis at 16.5 dpc and 0 dpp (Figure 1). However, like the newborn testis, the newborn ovary also exhibited a significant increase in Tgfbr3 mRNA on the day of birth compared to fetal ages (Figure 1).

To determine the cellular localization of TGFBR3 protein in developing gonads, immunofluorescent histochemistry was performed on gonad sections from several fetal and neonatal ages. Data from these analyses are summarized in Table I. To assess antibody specificity, WT and Tgfbr3 null fetal gonad sections were processed in parallel, and this analysis revealed no TGFBR3 immunostaining in the null gonad (Figure 2). In addition, in each assay, adjacent sections were processed with primary or secondary antibodies alone (data not shown) or with goat IgG as an isotype control (Figures 3F and 5F) in order to determine the specific signal over background. In the fetal testis from 12.5 to 16.5 dpc, TGFBR3 immunostaining was predominantly localized to cells in the coelomic epithelium and coelomic vessel (Figure 2A) and within the testicular interstitium (Figure 2A) and within the testicular interstitium (Figures 2A and 3A–E). Between 13.5 and 16.5 dpc, peritubular cells around the cords also showed TGFBR3 immunostaining (Figures 2A and 3B–C,E). Cells within the seminiferous cords of the fetal testis generally showed no immunostaining between

<table>
<thead>
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<th>12.5 dpc</th>
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<th>14.5 dpc</th>
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<td>Leydig cells</td>
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<td>Peritubular cells</td>
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<td>Granulosa cells</td>
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<td>Thecal cells</td>
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+ = low; ++ = moderate; +++ = high expression; – = not detected; NA = not applicable.
12.5 and 16.5 dpc (Figure 3A–E). At birth, the TGFBR3 expression pattern changed as TGFBR3 immunostaining became readily detectable within the seminiferous cords (Figure 3G–H). In the cords, TGFBR3 appeared to be localized to the Sertoli cell cytoplasm and to some of the gonocytes, spermatogonia, and spermatocytes from birth through 15 dpp (Figure 3G–L). By 15 dpp (Figure 3J–L), TGFBR3 expression within the Sertoli cells appeared reduced compared to neonatal levels. A subset of cells within the testicular interstitium expressed TGFBR3 throughout all postnatal ages examined, including large cells with the morphology of adult Leydig cells (Figure 3K).

To determine which cell lineages express TGFBR3 in the fetal testis, testis sections were processed in...
double labelling immunofluorescence assays using antibodies against TGFBR3 in conjunction with cell-type specific markers. This analysis revealed that in the fetal testis, TGFBR3 immunostaining did not colocalize the germ cell marker, MVH (Figure 4A–F). At 12.0 dpc, as the germ cells are first forming aggregates, TGFBR3 expression was localized to somatic cells in close association with MVH-positive germ cells (Figure 4A–C). Once the germ cells were enclosed inside the seminiferous cords (which can be seen as early as 12.5 dpc, see, e.g. Figure 4A) TGFBR3 immunostaining was detected nearly exclusively within peritubular cells and interstitial cells, with no TGFBR3 localizing to MVH-positive cells (Figure 4D–F). The interstitial cells staining positive TGFBR3 also stained positive for the Leydig cell marker, CYP11A1/P450-Scc (Figure 4G–I), the enzyme which catalyzes the conversion of cholesterol to pregnenolone in mitochondria of steroidogenic cells. In contrast to the fetal expression pattern, on the day of birth, TGFBR3 expression was detected in the Sertoli cell cytoplasm and within some of the MVH-positive gonocytes (Figure 4J–L), and this pattern of expression in both the somatic and germ cell lineages continued until 15 dpp (Figure 3I–L).

Tgfbr3 mRNA and protein expression during ovary morphogenesis

From 12.5 to 16.5 dpc, TGFBR3 immunostaining was present within ovarian somatic cells and in large cells with the rounded morphology of oogonia (Figure 5). Most of the immunostained oogonia were scattered throughout the ovary, but occasionally
several positive-staining oogonia were located close together in clusters (Figure 5A–C). In contrast, between the day of birth and 5 dpp, when germ cell cysts break down into primordial follicles in the mouse, virtually all of the oocytes expressed TGFBR3 (Figure 5D–E). Of the somatic cell lineages, the squamous granulosa cells of the neonatal primordial follicles did not express TGFBR3 (Figure 5E). However, differentiating granulosa cells in growing follicles expressed TGFBR3 (Figure 5G–I). TGFBR3 immunostaining was observed within granulosa cells as soon as they began to adopt the cuboidal shape that marked the transition of the primordial follicles into primary follicles and the onset of follicular growth (Figure 5G). Throughout the neonatal and pre-pubertal periods, oocytes and granulosa cells of primary, secondary and preantral follicles expressed TGFBR3, but little expression was localized to thecal cells during the developmental window examined (Figure 5H–I). Isolated cells within the ovarian interstitium showed moderate to low TGFBR3 expression at all postnatal ages examined (Figure 5D–I). MVH and TGFBR3 co-immunostaining was used to verify that cells of germ cell lineage expressed TGFBR3 in the fetal and neonatal mouse ovary (Figure 6A–L). At all fetal ages, only a subpopulation of oogonia and oocytes expressed TGFBR3 (Figure 6A–I), although at 13.5–14.5 dpc (Figure 6A–F) more oogonia and oocytes exhibited staining for TGFBR3 and MVH than at either 12.5 dpc (data not shown) or 16.5 dpc (Figure 6G–I). Virtually all oocytes from the day of birth (Figure 6J–L) onwards (data not shown) exhibited TGFBR3 and MVH co-localization.

Discussion

The current study demonstrates that Tgfr3 is differentially expressed in the male and female gonads during murine development. Tgfr3 transcript and protein levels increased with age in the male gonad while levels in the fetal ovary were relatively steady until the day of birth, when Tgfr3 expression significantly increased in both sexes. Tgfr3 was expressed in different cell lineages in male and female fetal gonad, suggesting that TGFBR3 has gender-specific roles during gonadal development. TGFBR3 has been demonstrated to be an accessory receptor for both TGFβ and inhibin, increasing the binding of these ligands to TGFβ and activin/BMP type II
receptors, respectively (Lopez-Casillas et al. 1993; Lewis et al. 2000; Wiater and Vale 2003). During gonadal development, TGFBR3 is coordinately expressed with its ligands and their type I and II receptors in specific gonadal cell populations, indicating that TGFBR3 may modulate both TGFβ- and inhibin-mediated processes. However, the in vivo roles of the TGFβs and inhibins during early gonadogenesis are poorly understood. Despite the presence of reproductive phenotypes in Tgfβ1 (Ingman et al. 2006; Ingman and Robertson 2007), Tgfβ2 (Sanford et al. 1997), and inhibin-α knockout mice (Matzuk et al. 1992), no detailed analyses of the fetal gonads in any of these mutants have been reported. The current results on Tgfr3 expression are discussed below relative to the literature on the expression patterns of the TGFBR3 ligands and their type I and II receptors and the in vitro actions of these growth factors in primary gonadal cultures.

TGFBR3 is dynamically expressed during testis development, with predominant expression first localized to the interstitial compartment of the fetal testis, and then shifting to the seminiferous cords at birth. The fetal testis interstitium and fetal Leydig cells, in particular, appear to be important targets for the TGFβs and activins, as type I and II TGFβ and activin receptors have been detected on these cell types in fetal rat and human testis (Olaso et al. 1998; Anderson et al. 2002). In the current study, TGFBR3 was localized to fetal Leydig cells during early testis development, and the increase in the level of TGFBR3 expression throughout fetal testis development was largely due to the increase in the number of TGFBR3-positive Leydig cells after 12.5 dpc. During fetal

Figure 6. Co-localization of TGFBR3 with a germ cell marker in ovary. Single confocal sections demonstrate TGFBR3 immunostaining (green fluorescence) in fetal (A–I) and neonatal (J–L) mouse ovary. Double labelling immunofluorescence was conducted with the germ cell marker MVH (red fluorescence) to demonstrate TGFBR3 co-localization (yellow fluorescence in C, F, I and L). Arrows indicate oogonia and oocytes which immunostain for both TGFBR3 and MVH. Asterisks, somatic cells. Scale bar = 50 μm and pertains to all images.
development, TGFBR3 may modulate both autocrine and paracrine actions of its ligands on fetal Leydig cells. All three TGFβ isoforms are expressed in the developing rodent testis, by fetal Leydig, Sertoli, germ and peritubular cells (for review, see Olaso and Habert 2000). Inhibin-α and βB subunits are also co-expressed beginning at 14.5 dpc in fetal rat Leydig and Sertoli cells, indicating that these cell types could produce inhibin B and activin B (Majdic et al. 1997). In vitro, TGFβs, activins, and inhibins have been reported to regulate Leydig cell proliferation and steroidogenesis, with the TGFβs acting to inhibit both processes in differentiated Leydig cells (Hsueh et al. 1987; Risbridger et al. 1989; Gautier et al. 1997; Olaso et al. 1999). As fetal Leydig cells are essential for the production of androgens which drive masculinization of the murine fetus (O’Shaughnessy et al. 2006), the function of TGFBR3 in fetal testis may impact upon the production of these key steroidal hormones and their downstream effects on the male reproductive tract.

TGFBR3 may also regulate the behavior of other fetal cell types during testis differentiation and maturation. Notably, the expression of TGFBR3 in the coelomic vessel and somatic cells closely associated with the forming cords from 12.0 to 14.5 dpc suggests that TGFBR3 may play a role in the establishment of fetal testis structure. Data from in vitro studies indicate that the TGFβs regulate somatic cell proliferation, death, migration, and aggregation in fetal testis (Skinner and Moses 1989; Gautier et al. 1997; Cupp et al. 1999; Konrad et al. 2000), as well as germ cell proliferation and survival (Olaso et al. 1998). Activated Smad2, a downstream signaling molecule of TGFβ and activin, was detected in both somatic and germ cell lineages in 12.5 dpc mouse gonad (Chuva de Sousa Lopes et al. 2005), suggesting that TGFβ and/or activin act on both lineages at the time of cord formation. TGFβ2 is of particular interest in fetal testis development because it is locally produced within rat testis Sertoli cells from 13.5 dpc onwards (Olaso and Habert 2000) and has been found to enhance aggregation of Sertoli cells in co-cultures with peritubular cells (Konrad et al. 2000). These data suggest that TGFβ2 may be a paracrine signal in fetal testis, originating from Sertoli cells and acting on TGFBR3-expressing interstitial and peritubular cells, to coordinate cell-cell interactions during testis morphogenesis. In support of this hypothesis, preliminary observations of the Tgfr3 null fetal gonads revealed that testis morphology is disrupted between 12.5 and 13.5 dpc (M.A. Sarraj, H.K. Chua, A. Umbers, K.L. Loveland, J.K. Findlay, and K.L. Stenvers, unpublished observations). However, TGFβ signalling receptors have only been detected on germ and Leydig cells in fetal testis (Olaso et al. 1998), and the mechanism by which the lack of Tgfr3 disrupts cord formation is not yet clear.

Although a direct role of TGFBR3 in testis cord formation is currently speculative, a 69-fold increase in Tgfr3 transcript levels has previously been reported in the adult testis of a seasonal-breeding species of mouse specifically during a period of rapid morphological change (Pyter et al. 2005).

TGFBR3 expression continues in the interstitial compartment of the testis during the neonatal period, with sustained high expression by Leydig cells. In addition, TGFBR3 becomes highly expressed within the seminiferous cords at birth. These findings are in accordance with a previous study which detected transcripts encoding all three TGFβ receptor subtypes in 10–90 dpp rat testis by Northern blot analysis, with the highest expression detected in immature testis in Leydig-enriched, Sertoli, peritubular and germ cell fractions (Le Maguereuse-Battistoni et al. 1995). The coincident expression of all three TGFβ receptor subtypes by multiple cell types in postnatal testis suggests that the TGFβs are important regulators of postnatal testis growth and maturation. TGFBR3 may directly contribute to the regulation of spermatogenesis, as gonocytes express TGFβ type I–III receptors and the TGFβs regulate mitosis, death, and differentiation of gonocytes in vitro. Rodent gonocytes are quiescent during late gestation but re-enter the cell cycle around 1–3 dpp. In cultures of rat testis fragments, these mitotically active gonocytes undergo apoptosis in response to TGFβ (Olaso et al. 1998) while activin increases the number of gonocytes directly after birth and inhibits gonocyte differentiation into spermatogonia (Meehan et al. 2000). Apoptosis is postulated to contribute to the morphogenesis of the seminiferous cords by ensuring that germ cell numbers are appropriately balanced to the number of supporting Sertoli cells (for review, see Itman et al. 2006). TGFBR3 may contribute to the negative control of germ cell number and the promotion of germ cell maturation as the presence of this receptor on postnatal gonocytes would be anticipated to affect both TGFβ- and (via inhibin) activin-mediated processes.

Since Sertoli cell number directly impacts on germ cell survival and maturation, TGFBR3 may also influence spermatogenesis by regulating the actions of its ligands on Sertoli cells in postnatal testis. Type I and II receptors for TGFβ and activin have been reported on neonatal and pre-pubertal Sertoli cells in rat (Le Magueresse-Battistoni et al. 1995; Buzzard et al. 2003), and the current study has shown that TGFBR3 is abundantly expressed in immature mouse Sertoli cells during a window of their development from birth until 15 dpp. During this phase of development, immature Sertoli cells are rapidly proliferating. An apparent decrease in TGFBR3 expression in Sertoli cells at 15 dpp coincides with the cessation of proliferation and the onset of Sertoli cell maturation and spermatogenesis (for review, see
Loveland and Robertson 2005). This suggests that TGFBR3 is particularly important to immature Sertoli cell biology but not to maturing or differentiated Sertoli cells. This conclusion is in agreement with studies which have failed to detect Tgfbr3 expression in adult Sertoli cells (Lewis et al. 2000; MacConell et al. 2002; Konrad et al. 2006).

In contrast to the developing testis, TGFBR3 was detected in both germ cell and somatic cell lineages in mouse ovary throughout fetal and neonatal development. This indicates that TGFBR3 plays different roles in ovary than in testis, particularly in the germ cell lineage. Reports are conflicting as to whether inhibins are produced in fetal ovary (Majdic et al. 1997; Kang et al. 2003; Weng et al. 2006). However, TGFβ1 and TGFβ2 have been detected by immunohistochemistry in rat ovarian somatic cells from 14.5 dpc through gestation (Levacher et al. 1996). In addition, activated Smad2 was detected in the 12.5 dpc murine gonad in both germ cell and somatic cell lineages, suggesting that TGFβ may act on both lineages (Chuva de Sousa Lopes et al. 2005). This conclusion is supported by the expression of the type I and II TGFβ receptors and the three TGFβ isoforms in both germ and somatic cell populations in human fetal ovary (Schilling and Yeh 1999). A recent study showed that TGFβ1 and TGFβ2 inhibit somatic cell proliferation in embryonic chick ovary organ cultures without affecting oogonia proliferation or death, although the migration of germ cells was altered (Mendez et al. 2006). In mice lacking the type I TGFβ receptor, PGC migration was altered but not PGC proliferation or death (Chuva de Sousa Lopes et al. 2005), suggesting that the TGFβs may play similar roles in the fetal chick and murine ovary, regulating somatic cell proliferation and germ cell migration. The presence of TGFBR3 on both somatic and germ cells in fetal ovary suggests TGFBR3 could serve as a TGFβ accessory receptor on both oogonia and somatic cells. However, preliminary examination of the Tgfbr3 null ovary indicates no overt morphological phenotype at 12.5–14.5 dpc, indicating that TGFBR3 may not play an essential role in establishing ovarian structure at this time (M.A. Sarraj, H.K. Chuva, A. Umbers, K.L. Loveland, J.K. Findlay, and K.L. Stenvers, unpublished observations).

In the newborn mouse ovary, the level of Tgfbr3 expression increased and was detected predominantly in oocytes. Perinatally, the oocytes are associated in germ cell cysts, which are in the process of breaking down to form individual primordial follicles (Pepling and Spradling 2001). This process involves the encapsulation of oocytes with somatic (pre-granulosa) cells, and those oocytes that do not successfully form primordial follicles undergo apoptosis (Pepling and Spradling 2001). Few studies have examined the TGFβ superfamily during this process of neofolliculogenesis. However, recently, activin A was shown to significantly increase the number of primordial follicles generated during this period, a process linked to an activin-mediated increase in germ cell and somatic cell proliferation (Bristol-Gould et al. 2006). In addition, in human fetal ovaries, both the type I and II TGFβ receptors, as well as all of the TGFβ isoforms, are highly expressed in oocytes and moderately expressed in supporting granulosa cells during the second trimester (14–24 weeks) of gestation (Schilling and Yeh 1999) when neofolliculogenesis is occurring in humans (Forabosco and Sforza 2007). These studies suggest that the TGFβs may also regulate the process of follicle formation; TGFBR3 on oocytes may be required for TGFβ or inhibin to optimally counterbalance the stimulatory effects of activin on this process.

After primordial follicle formation, a subset of the primordial follicles begins to grow and differentiate in the human fetal (Forabosco and Sforza 2007) and rodent neonatal periods (Drummond et al. 2003; Kerr et al. 2006). Crosstalk between oocytes and granulosa cells is believed to coordinate the differentiation, growth, and maturation of ovarian follicles, with multiple members of the TGFβ superfamily taking part in the regulation of these processes (for review, see Juengel and McNatty 2005). All components of the TGFβ and activin/inhibin signalling systems are expressed during the mammalian postnatal period, although there are cell-, age- and species-specific differences (for review, see Drummond et al. 2003; Juengel and McNatty 2005). In the neonatal period, granulosa cells may be the direct targets of TGFβ, activin and inhibin. The type I and II activin and TGFβ receptors have been detected on granulosa cells in postnatal rodent ovary, and the TGFβs negatively regulate proliferation and steroidogenesis in these cells in vitro (Drummond et al. 2002; Juengel and McNatty 2005). TGFBR3 on both oocytes and granulosa cells may contribute to the regulation of follicle maturation during this period of development. TGFBR3 immunostaining was intense in oocytes, from the day of birth. TGFBR3 expression was also detected at moderate levels on granulosa cells throughout the postnatal period examined. Notably, TGFBR3 was not detected on the squamous granulosa cells of the primordial follicles but on the cuboidal granulosa cells of growth-initiated follicles from the time of their differentiation, suggesting that TGFBR3 may take part in granulosa cell differentiation and maturation. Of interest, Tgfbr3 mRNA expression is increased by follicle stimulating hormone in human granulosa-luteal cell cultures (Liu et al. 2003) and synergistically by follicle stimulating hormone and estradiol in rat granulosa cell cultures (Omori et al. 2005), suggesting that TGFBR3 expression on granulosa cells is regulated by pituitary- and ovarian-derived hormones during folliculogenesis. However, unlike previous studies in developing and adult rat (Lewis et al.
2000; Drummond et al. 2002; MacConell et al. 2002) and adult human (Liu et al. 2003), very little TGFBR3 immunostaining was detected in the thecal layer during the murine neonatal/prepubertal period. This suggests that the role of Tgfr3 in these species differs during neonatal theca development and that, unlike in the rat and human, TGFBR3 is not necessary to modulate the effects of the TGFBs and of inhibins on thecal cell biology in neonatal/prepubertal growing follicles in mice.

In summary, this is the first report to characterize Tgfr3 mRNA and protein expression throughout male and female murine gonadogenesis. The current study demonstrates that Tgfr3 mRNA and protein are differentially expressed in the male and female gonads during murine development, suggesting multiple roles for this receptor in the developing gonad of both sexes. Notably, we observed prominent expression of Tgfr3 during the critical periods of development that determine the reproductive capacity of the adult mouse. This gives support to the notion that the reproductive defects observed in the adult Tgfr3 null mice (Stenvers et al. 2003) may have their origin in the fetal or neonatal periods of gonadogenesis. Fetal gonadogenesis in the Tgfr3 null mouse line warrants further study and will be the future focus of our work.

Acknowledgements

This work was supported by the NHMRC of Australia (RegKeys 338516 and 198705 for KLS, JKF; 384108 for KLL) and the Australian Research Council (KLL). The technical assistance of Marnie Sparrow is gratefully acknowledged.

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