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Transforming Growth Factor Beta (TGFβ1, TGFβ2 and TGFβ3) Null-Mutant Phenotypes in Embryonic Gonadal Development

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Abstract

The role transforming growth factor beta (TGFβ) isoforms TGFβ1, TGFβ2 and TGFβ3 have in the regulation of embryonic gonadal development was investigated with the use of null-mutant (i.e. knockout) mice for each of the TGFβ isoforms. Late embryonic gonadal development was investigated because homozygote TGFβ null-mutant mice generally die around birth, with some embryonic loss as well. In the testis, the TGFβ1 null-mutant mice had a decrease in the number of germ cells at birth, postnatal day 0 (P0). In the testis, the TGFβ2 null-mutant mice had a decrease in the number of seminiferous cords at embryonic day 15 (E15). In the ovary, the TGFβ2 null-mutant mice had an increase in the number of germ cells at P0. TGFβ isoforms appear to have a role in gonadal development, but interactions between the isoforms is speculated to compensate in the different TGFβ isoform null-mutant mice.

Keywords

Testis; Ovary; Knockout; TGF-beta 1; TGF-beta 2; TGF-beta 3; Embryonic Development

INTRODUCTION

Gonadal development during sex determination requires a coordinated somatic and germ cell differentiation and proliferation during the embryonic period. In the mouse, crucial morphogenic events are initiated in the testis at embryonic day 11.5 (E11.5; plug day considered to be E0) [1]. At this stage of male gonadal development, SRY expression is initiated in the precursor Sertoli cells and male sex determination ensues. In the developing testis at E12.5 the pre-peritubular cells migrate from the adjacent mesonephros to enclose precursor Sertoli and germ cell aggregates to promote development of cords. Testis seminiferous cords can be identified by E14 in rats. After seminiferous cord formation, somatic and germ cells

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Summary Sentence - TGF-β isoforms have an important role in embryonic gonadal development, but isoforms appear to compensate for each other.

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undergo the highest level of cellular proliferation that occurs during testis development [2]. In the rodent ovary there is no major morphological alteration until after birth when primordial follicles assemble [3]. During ovarian sex determination the germ cells initially proliferate and then enter meiosis and arrest in prophase I to form clusters (i.e. nests) of germ cells. These nests of germ cells after birth degenerate through random apoptosis of selected germ cells to form several primordial follicles from each nest. The somatic cells that surround the nests of meiotically arrested germ cells form the precursor granulosa cells associated with individual oocytes to form the primordial follicles after birth. The current study was designed to investigate the late embryonic testis and ovary morphology at embryonic day 15 (E15) and at birth, postnatal day 0 (P0), in TGF β null-mutant mice.

Transforming growth factor beta's (TGF β s) are critical for growth, differentiation and development of many different cell types within an organism. Five TGF β isoforms have been identified with three (TGF β 1, TGF β 2, TGF β 3) present in mammals [4-6]. The TGF β isoforms are encoded from individual genes located on different chromosomes. The primary functions of the TGF β s include enhancing formation of the extracellular matrix, regulation of cellular differentiation, and inhibition of proliferation of most cells [6,7]. Inhibition of growth by TGF β s occur through an arrest of the cell cycle in late G1 phase and requires actions on the retinoblastoma protein and cyclin-dependent kinases. The effects of TGF β s are mediated through activation of the membrane receptors TGF β -receptor I (TGF β RI) and TGF β -receptor II containing serine/threonine kinase activity [7,8], with all TGF β isoforms binding to TGF β RII [9].

TGF β isoforms display similar, although not identical, biological activity and differential tissue expression [10-12]. For example, in the mature testis TGF β 3 is the major isoform expressed [13,14]. TGF β 1 is expressed by the Sertoli cell [15,16] and may be important for spermatogenesis [17]. Sertoli cell production of TGF β 1 may be targeted to the germinal cell population due in part to the blood-testis barrier [18]. All 3 isoforms (TGF β 1, TGF β 2 and TGF β 3) are expressed in the male gonad and their receptors are present in the testis in both somatic cells and germ cells [9,18-21]. The embryonic (E14) testis has TGF β 1 and TGF β 2 expression by Sertoli cells, germ cells and selected interstitial cells, while TGF β 3 is expressed at high levels at the testis and mesonephros interface by precursor peritubular cells [9].

Gene knockout null-mutant and over-expression experiments with TGF β have demonstrated that precise regulation of each isoform is essential for survival. The null-mutant TGF β 1 embryos show defects in vasculogenesis and hematopoiesis and often die around birth. However, some TGF β 1 knockouts can be phenotypically normal until approximately 3 weeks after birth and then develop a severe wasting syndrome related to excessive inflammatory responses [22,23]. Reproductive tracks and organs within TGF β 1 knockout mice have been studied [24]. Significant deviations from normal Mendelian ratios are observed with decreased offspring for both heterozygotes and homozygotes of TGF β 1 null-mutants. TGF β 2 knockout mice show multiple developmental defects including cardiopulmonary, skeletal, ocular, and urogenital systems defects [25-27]. Urogenital defects include ectopic and hypoplastic testis. TGF β 2 knockout mice generally die by birth. TGF β 3 knockout mice show delayed pulmonary and defective palate development, and die prenatally due to failure to suckle [28,29]. Although knockout mice for each TGF β isoform have been generated, all animals generally die at the neonatal stage making it difficult to investigate the significance of TGF β s in gametogenesis in the adult gonads. Double knockout null mutants of combinations of TGF β isoforms die early in embryonic development prior to gonadal development [30]. Although functions have been found for TGF β family members in the adult testis and ovary [31-34], studies are needed with conditional knockouts to pinpoint the physiological significance of TGF β s null mutants in the postnatal gonad [35].

The current study was designed to investigate the role of TGFbs in late embryonic testis and ovary development using knockout null-mutant mice. To determine actions of TGFb on testis development, two time points were evaluated. The first was E14-E15 after seminiferous cord formation occurs and the second was P0 when cells of the testis are actively proliferating. To evaluate the role of TGFb on ovarian development, ovaries from P0 mice were collected, when oocytes are starting to assemble into primordial follicles. The hypothesis tested was that TGFb1, TGFb2, and TGFb3 have critical roles in embryonic gonadal development and are necessary for normal cell-cell interactions during the process of testis and ovary morphogenesis.

MATERIALS & METHODS

Animals

TGFb1 mouse (strain 129) and TGFb2 and TGFb3 mouse (C57black6) heterozygous (+/-) mice were bred to generate TGFb1, TGFb2 and TGFb3 knockout homozygous (-/-) mice, respectively. The testes from TGFb1, TGFb2 and TGFb3 (-/-), (+/-) and wild type (+/+) embryos were collected from timed pregnant mice at embryonic day 14-15 (E14-E15, E0 = plug date). The testes and ovaries were collected from TGFb1, TGFb2 and TGFb3 pups at P0-P1 (P0 = postnatal day 0). When animals survived the testes from TGFb1 (-/-), (+/-) and (+/+) pups were collected at P10-P20. Tails from embryos and the pups were collected for genotyping. All protocols were approved by the Washington State University Animal Care and Use Committee.

Genotyping and SRY Analysis

Tail fragments were collected and genomic DNA was isolated by procedures previously reported [2,9]. Briefly, the tails were digested with proteinase K (0.15 mg/ml) overnight at 55°C, followed by precipitation with saturated NaCl and 100% ethanol, and re-suspended in sterile water. Approximately 100 ng of genomic DNA was used for PCR analyses. For TGFb1 analyses, 30 cycles of 94C/30s, 58C/30s and 72C/90s using forward 5'-GAGAAGAAGTGTCTGTGTGCG 3' and reverse 5'-GTGTCCAGGCTCCAAATATAG 3' primers containing 1.2% DMSO were used per reaction. For TGFb2 analyses, 30 cycles of PCR was performed at 94C/30s, 55C/30s and 72C/90s using forward 5'-CTCCATAGATATGGGCATGC and reverse 5'-AATGTGCAGGATAATTGCTGC primers containing 1.2% DMSO per reaction. For TGFb3 analyses, 30 cycles of PCR was performed at 94C/30s, 58C/30s and 72C/90s using forward-1 5'-TGGGAGACATGGCTGTAAGT and forward-2 5'-CACTCACACTGGAAGTAGT and reverse 5'-GATGGGATGTTTGGTTGGT primers per reaction. SRY analysis was utilized to confirm sex of the embryos using the PCR conditions previously reported [2].

Semi-quantitative PCR Analysis

RNA was extracted from testis sections on slides with the use of Trizol reagent (Sigma, St. Louis, MO). The TGFb1, TGFb2, and TGFb3 null mutant gonadal RNA was reverse transcribed with 3' TGFb isoform specific primers and then the PCR reaction performed with similar primers as listed above except for the TGFb2, which used forward 5'-CAGGAGTGGCTTCACCACAAAG and reverse 5'-TGGCATATGTAGAGGTGCCATCA, and the TGFb3, which used forward 5'-TCGACATGATCCAGGGACTG and reverse 5'-CCACTGAGGACACATTGAAACG primers per reaction. A constitutively expressed S2 gene expression was used to normalize the data with the use of forward 5'-TGCCAGTGCAGAAGCAGACT and reverse 5'-CACCAAGACCAACGTGACCA primers in the same RT-reaction. TGFb isoform specific products were extracted and sequenced to confirm the identify of the PCR product. PCR electrophoretic band intensity was measured with a scanner to quantify the data.

Histology

The testes and ovaries were fixed in Bouin's fixative (Sigma, St. Louis, MO) for 2 hrs, washed in 70% ethanol and embedded in paraffin using standard procedures. Serial ribbon sections from each testis and ovary were stained with hematoxylin and eosin (H&E) using standard procedures for morphological analyses. The Center for Reproductive Biology Histology Core Laboratory assisted with this analysis.

Morphological Analysis

Testis and ovary sections from genotyped animals at each developmental age were analyzed as described earlier [36]. Briefly, the testes sections were evaluated for the presence of seminiferous cords, area of seminiferous cords, and area of the interstitium. Using computer aided morphometry seminiferous cords were circled within a section to calculate the total area of seminiferous cords (mm^2). The seminiferous cord area was then subtracted from the total testis area to determine the interstitial area of each section. The data for each averaged area (for a particular genotype) were depicted as the number of mm^2 per designated testis area (mm^2). Because the sections were relatively small, serial sections on each slide were utilized to obtain these measurements. Therefore, for each genotype represented, at least six sections from each testis were utilized to obtain the cord area, interstitial area, and number of seminiferous cords. All morphological observations were normalized per a defined testis section area (mm^2) to correct for variation in testis and section size,

Germ Cell Nuclear Antigen (GCNA) Staining

Germ Cell Nuclear Antigen (GCNA) staining was analyzed as previously described [37]. Briefly, the testis and ovary sections were deparafinized and rehydrated through a series of alcohols. Sections were boiled in a microwave oven for 15 min in sodium citrate buffer for antigen retrieval and blocked in 10% calf serum for 30 min at room temperature. The sections of testes and ovaries were then incubated with GCNA1 primary antibody (1:10 dilution) at 4C for 16 hrs. The sections were then incubated at 20C for 2 hrs with biotinylated goat anti-mouse secondary antibody (1:300; Vector Laboratories, Burlington, CA). Secondary antibody was detected using the Histo stain-Sp Kit (Zymed Laboratories, San Francisco, CA). At least two experiments were conducted using GCNA1 antibodies at each developmental time. In each experiment, 3 serial sections of 4-5 testes and ovaries for each developmental age were analyzed.

Testicular Cell Apoptosis

The Fluorescein In Situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN) was utilized to detect apoptosis of testicular cells as described earlier [38]. The assay measures fragmented DNA from apoptotic cells by catalytically incorporating fluorescein-12-dUTP at the 3'DNA ends using the enzyme terminal deoxynucleotidyl transferase (TdT), which forms a polymeric tail using the principal of the TdT-mediated dUTP Nick-End Labeling (TUNEL) assay. All the fluorescent cells in each testis section were counted at 100X magnification. In each experiment, 3 serial sections of 4-5 testis and ovaries for each developmental age were analyzed. The average number of fluorescent cells/whole testis was calculated.

Microarray Analysis

RNA was collected from E13, E14, E16, P0 and P6 testes and E13, E14 and E16 ovaries from Sprague-Dawley rats as previously described [39]. RNA was hybridized to the Affymetrix (Affymetrix, Santa Clara, California) rat 230A gene chip. The Genomics Core Laboratory of the Center for Reproductive Biology at Washington State University performed the analysis as previously described [40,41]. Briefly, RNA from the cells was reverse transcribed into cDNA then was transcribed into biotin labeled RNA. Biotin labeled RNA was then hybridized

to the Affymetrix rat 230A gene chips. Each gene set is composed of 11 pairs of 24-mer oligonucleotides, with one anti-sense strand specific for the gene and one anti-sense strand with single point mutations for use as comparative negative control. Biotin labeled RNA was then visualized by labeling with phycoerythrin-coupled avidin. The microarray was scanned on a Hewlett-Packard Gene Array Scanner (Hewlett-Packard Co., Palo Alto, CA). Two different microarray chips from two different RNA samples involving different groups of animals were analyzed for each E13, E14, E16, P0 and P6 testis and E13, E14 and E16 ovary samples. The microarray data set can be observed at www.skinner.wsu.edu and was submitted to GEO. Transcript levels from mouse microarray analyses of E11.5, E12.5, E16.5, P0 and P6 testis and ovary samples were extracted from previously reported data sets [39].

Statistical Analysis

Data were analyzed with GraphPad Prism (GraphPad Prism Software, Inc., San Diego, CA). All values are expressed as the mean $\pm$ SEM of the parameter measured. The n value was based on different testis and ovary and not replicates from the same gonad. Each tissue section analysis involved a minimum of 6 different areas. Statistical analysis was performed using one-way ANOVA followed by post-hoc test using Dunnett test for comparison to controls and the Tukey-Kramer honestly significant difference test for multiple comparisons between different treatment groups. Statistical difference was confirmed at $P < 0.05$. Specific comparisons, analyses, replicates and results are presented in the different figure legends.

RESULTS

The gene expression of TGFb1, TGFb2 and TGFb3 isoforms and their receptors were analyzed by microarray analyses for E13-P6 rat testes, E11.5-P6 mouse testes, E13-E16 rat ovaries and E11.5-E16.5 mouse ovaries, Table 1. TGFb1 mRNA was below the detection limits in both rat and mouse samples studied. TGFb2 mRNA levels increased from E14-P0 and then decreased at P6 in the rat testis, Table 1A. TGFb2 mRNA levels were constant in the mouse testes development with the highest level of expression at E16.5, Table 1B. TGFb3 mRNA levels gradually increased from E13-E16 during the rat testis development, then slowly decreased in the postnatal samples. TGFb3 had a consistent increase until P6 during mouse testis development, Table 1B. In the rat testis, TGFbRI had consistently low expression during testis development, Table 1. TGFbRII expression levels were high during testes development with the highest expression detected in the P6 sample, Table 1A. TGFbRIII expression increased from E13-P0 then decreased in the P6 samples, Table 1. In the mouse testis, the expression of TGFbRI, TGFbRII and TGFbRIII were low during all time points, Table 1B. In general the TGFb's pattern of gene expression were similar or conserved during mouse and rat testis development. In contrast, the TGFb receptors pattern of gene expression were distinct between rat and mouse.

In the rat ovary samples, the TGFb1 mRNA levels were below detection in the E13-E16 samples, Table 1A. TGFb2 and TGFb3 mRNA levels were consistent in the E13-E16 samples. The expression of TGFbRI and TGFbRIII were low in the E13-E16 ovary samples. TGFbRII was the most abundant of the three receptors in the ovary and it remained consistent between E13-E16, Table 1A. In the mouse ovary samples, the TGFb1 mRNA levels were below detection in the E11.5-E16.5 samples, Table 1B. TGFb2 mRNA levels were consistent in the E11.5-E16.5 samples. The TGFb3 mRNA levels gradually increased from E11.5-E16.5. Similar to the rat ovary samples, the expression of TGFbRI and TGFbRII were low in the E11.5-E16.5 samples and the TGFbRIII was the most abundant of the three receptors in the mouse ovary and remained consistent between E11.5-E16.5, Table 1B. As seen in the testis, the ovarian TGFb expression patterns were similar between rat and mouse with the TGFb receptor expression being distinct.

The gene expression of the TGFb isoforms was assessed in RNA isolated from P0 testis sections to confirm that alternate TGFb isoforms were expressed in the individual isoform null mutant mice. RT-PCR data was normalized with the constitutively expressed S2 gene to provide a semi-quantitative analysis. The identity of PCR products was confirmed with DNA sequence analysis. The specific isoform null mutants had no expression of that TGFb isoform. The alternate TGFb isoforms were expressed in each of the TGFb1, TGFb2 or TGFb3 null mutant P0 testis samples (data not shown). No major change in expression levels was observed when compared to control wild-type P0 testis samples (data not shown).

To generate TGFb null-mutant homozygote (-/-) animals the TGFb1, TGFb2 and TGFb3 heterozygous (+/-) females were housed with age matched (+/-) males and examined every day for the presence of vaginal plugs. The plug day was considered embryonic day 0 (E0). Forty litters from TGFb1 (+/-) breedings, 38 litters from TGFb2 (+/-) breedings and 26 litters from TGFb3 (+/-) breedings were examined. The average TGFb1 litter size was 2.95 ± 0.29 female pups and 3.35 ± 0.29 male pups. The average TGFb2 litter size was 3.35 ± 0.35 females and 3.19 ± 0.25 males pups. The average TGFb3 litter size was 3.4 ± 0.43 females and 3.8 ± 0.41 males pups. There was no difference in pup sex ratio between TGFbs (+/-) breedings, Figure 1A. The control wild type 129 and C57BL/6 mice strains have litter sizes of 6.0 and 6.6, respectively (Jackson Labs, Bar Harbor, Maine), so the TGFb (+/-) breeding litters were smaller in size to the control wild type.

The compiled genotype ratios for TGFb1 were 2.18 ± 0.29 , 3.10 ± 0.32 , and 1.03 ± 0.17 for (+/+), (+/-) and (-/-), respectively. The compiled TGFb2 genotype ratios were 3.0 ± 0.37 , 3.3 ± 0.33 , and 0.32 ± 0.11 for (+/+), (+/-) and (-/-), respectively. The genotype ratios for TGFb3 were 2.30 ± 0.44 , 4.08 ± 0.51 , and 1.12 ± 0.22 for (+/+), (+/-) and (-/-), respectively. Figure 1B presents the data as percentages of the total litter. The average genotype ratios were significantly different for TGFb1 ($p < 0.0001$), TGFb2 ($p < 0.0001$) and TGFb3 ($p < 0.0025$), based upon the observed and expected Mendelian ratio, Figure 1B. Observations demonstrate significant embryonic loss for homozygote (-/-) mutants for all TGFb isoforms.

The testes morphology was examined at E15 and P0 for wildtype (+/+), heterozygote (+/-) and homozygote (-/-) samples from TGFb1, TGFb2 and TGFb3 mice. The gross morphology of the testes from the (-/-) mice was similar to the (+/-) and (+/+) for TGFb1, TGFb2 and TGFb3 mice. Representative micrographs for TGFb2 (+/+), (+/-) and (-/-) E15 testes are shown in Figure 2A-C. Similar to the E15 testis, the gross testis morphology at P0 was also similar between the (+/+), (+/-) and (-/-) TGFb1, TGFb2 and TGFb3 mice. Representative micrographs for TGFb1 (+/+), (+/-) and (-/-) P0 testes are shown in Figure 2D-F. The ovary morphology was examined at P0 for (+/+), (+/-) and (-/-) samples from TGFb1, TGFb2 and TGFb3 mice. As observed with the testis, no gross morphological differences were found in the ovaries (data not shown).

Testis morphology was analyzed in more detail in various samples. Testis and testis section size was variable so all morphological data was normalized per defined testis section area (mm^2) to correct for variation in size. The number of seminiferous cords per mm^2 testis area for E15 embryos were determined for (+/+), (+/-) and (-/-) TGFb1, TGFb2 and TGFb3 mice, Figure 3A. There was a significant ($p < 0.01$) decrease in the number of seminiferous cords between TGFb2 (-/-) and (+/-) testes and the (+/+) testes. No significant difference was observed between TGFb2 (-/-) and (+/-) testes. No significant difference in the number of seminiferous cords per mm^2 testis area for E15 embryos was present for either TGFb1 or TGFb3 animals, Figure 3A. The number of seminiferous cords per mm^2 testis area for P0 pups from TGFb1, TGFb2 and TGFb3 mice was determined. No significant difference was observed in the number of seminiferous cords per mm^2 testis area for P0 pups from TGFb1, TGFb2 nor TGFb3 animals, Figure 3B.

The area of the seminiferous cords per mm² testis area for E15 embryos and P0 postnatal pups were determined for (+/+), (+/-) and (-/-) TGFb1, TGFb2 and TGFb3 animals, Figure 4. No significant difference was found in area of seminiferous cords per mm² for E15 testes between (-/-), (+/-) and (+/+) for TGFb1, TGFb2 or TGFb3, Figure 4A. Similar to the E15 seminiferous cord analyses, there was no significant difference in the area of seminiferous cords per mm² testis area for P0 pups from (+/+), (+/-) and (-/-) TGFb1, TGFb2 or TGFb3 animals, Figure 4B.

The number of developing germ cells per mm² testis area for E15 and P0 samples were determined for (+/+), (+/-) and (-/-) TGFb1, TGFb2 and TGFb3 mice, Figure 5. Figure 5A-C are representative micrographs of the GCNA staining of E15 testis cross-sections from (+/+), (+/-) and (-/-) TGFb1 embryos. There was a significant ($p < 0.05$) increase in germ cell number between TGFb1 (+/+) and (+/-) E15 testes, Figure 6A. No significant differences in germ cell numbers were observed for the (+/+), (+/-) and (-/-) TGFb2 and TGFb3 mice, Figure 6A. The number of developing germ cells per mm² testis area for P0 samples were determined for (+/+), (+/-) and (-/-) TGFb1, TGFb2 and TGFb3 mice. Figure 5D-F are representative micrographs of the GCNA staining of P0 testis cross sections from (+/+), (+/-) and (-/-) TGFb1 animals. There was a significant ($p < 0.05$) decrease in developing germ cell numbers between TGFb1 (-/-) testes and the (+/-) and (+/+) testes, Figure 6B. No significant difference in germ cell numbers was observed for the (+/+), (+/-) and (-/-) TGFb2 and TGFb3 animals, Figure 6B.

The number of apoptotic cells per mm² testis area for E15 embryos were determined for (+/+), (+/-) and (-/-) TGFb1, TGFb2 and TGFb3 mice. Figure 7A and B are representative micrographs of the TUNEL staining of E15 testis cross-sections from (+/+) and (-/-) TGFb2 embryos. The majority of the positively labeled cells were outside the seminiferous cords indicating germ cells may not be affected at this time, Figure 7. There was an apparent increase in apoptotic cell number in the TGFb2 (+/-) and (-/-) samples compared to the (+/+) samples, however, this increase was only statistically significant for (-/+), Figure 8A. There was no significant difference in apoptotic cell numbers in the testis of E15 embryos from (+/+), (+/-) and (-/-) TGFb1 and TGFb3 mice, Figure 8A. The number of apoptotic cells per mm² testis area for P0 samples were determined for (+/+), (+/-) and (-/-) TGFb1, TGFb2 and TGFb3 mice. Figure 7C and D are representative micrographs of the TUNEL staining of P0 testis cross-sections from (+/+) and (-/-) TGFb2 mice. As with the labeling in the E15 testis sections, the majority of the labeling was located outside the seminiferous cords. There was a significant ($p < 0.05$) increase in apoptotic cell number between the TGFb3 (+/-) testis and the (+/+) and (-/-) testis samples, Figure 8B. No significant difference in number of apoptotic cells was found for (+/+), (+/-) and (-/-) TGFb1 or TGFb2 P0 testis, Figure 8B.

The homozygote (-/-) TGFb2 and TGFb3 animals all died shortly after birth or during embryogenesis. However, a small percentage of the TGFb1 (-/-) pups survived after birth up to 20 days. At postnatal day 20 (P20) the testicular size of TGFb1 (-/-) animals appeared 30-40% smaller than their litter mate (+/+) and (+/-) animals. Analyses of apoptotic cells in these (+/+), (+/-) and (-/-) TGFb1 P10 to P20 testes demonstrated an increase in TUNEL positive labeling inside the seminiferous tubules (i.e., germ cell labeling) in the (-/-) mice compared to the (+/+) and (+/-) animals (data not shown).

Ovary morphology was also investigated in more detail. Analyses of the ovaries of P0 (+/+) and (-/-) TGFb1, TGFb2 and TGFb3 mice is shown in Figure 9. Representative micrographs of the morphology of the ovaries and GCNA staining for (+/+) and (-/-) TGFb2 P0 animals are presented. The number of developing germ cells per mm² of ovarian area for P0 samples was determined for (+/+), (+/-) and (-/-) TGFb1, TGFb2 and TGFb3 animals, Figure 9E. There was an increase in germ cells in the homozygote (-/-) samples compared to the (+/+) samples for

TGFb2. No significant difference was observed in the number of germ cells for (+/+) and (-/-) TGFb1 or TGFb3 samples, Figure 9E.

DISCUSSION

The transforming growth factor-beta family of growth factors has been shown to be important for testis and ovary function at a number of stages of development [31-34,42,43]. This large family of growth factors involves the bone morphogenic proteins (BMP's), growth and differentiation factors (GDF's), activins, inhibins and the TGF isoforms [44]. The TGFb1, TGFb2, TGFb3 isoforms are critical to regulate cell growth, differentiation and development. This involves growth inhibition and extracellular matrix production [4]. In the testis, the TGFbs are localized to most cell types, as well as the TGFb receptor isoforms [45]. The expression of TGFb isoforms in the testis alters during development and can be influenced by other hormones and growth factors, as well as physiological conditions [46,47]. An example of the actions of TGFbs in the testis is the ability to integrate TGFb signal transduction events with other signaling pathways to influence extracellular matrix, junction complex proteins and the blood-testis barrier [48,49]. Another example of TGFbs actions in the testis is the ability to regulate germ cell apoptosis and spermatogenesis [50,51]. In the ovary, TGFbs have a role in cellular proliferation and differentiation associated with preantral and antral follicle development [31-34]. The current study was designed to investigate the role of TGFb isoforms in the embryonic and early postnatal testis and ovary using null-mutant (i.e. knockout) mouse models.

Gene knockout null-mutant mice have been generated for each TGFb isoform. Due to the importance of TGFb to all tissues these null-mutants will generally die late embryonically or early postnatally. Therefore, these TGFb knockout models are not useful to investigate pubertal or adult testis function, but can be used to investigate embryonic gonadal development and function. Unfortunately, double knockout TGFb null-mutants of various combination of isoforms die early in embryonic development prior to gonadal development [30]. Therefore, the combined functions of the different TGFb isoforms could not be analyzed with double knockout mice. The current study examines the gonadal phenotype of the individual TGFb1, TGFb2 and TGFb3 knockouts at embryonic day 15 (E15) after cord formation in the testis and at the day of birth, postnatal day 0 (P0). The background mouse strain was 129 for TGFb1 and C57BL/6 for TGFb2 and TGFb3. The phenotypes observed can be influenced by the background strain, so should be considered in any data interpretations.

The sex ratio of the different TGFb isoform knockouts was not affected demonstrating no preferential sex specific effect on embryonic survival or death. In contrast, the genotype of homozygotes (-/-) for all the TGFb1, TGFb2 and TGFb3 knockouts demonstrated a non-Mendelian distribution and suggests an apparent embryonic loss of some homozygote (-/-) embryos. The reduced number of (-/-) null-mutant animals required a large number of litters to be obtained such that sufficient numbers of animals could be analyzed.

Previous analysis has localized TGFb isoform expression in the developing embryonic testis [9]. Immunohistochemical localization of TGFb1 and TGFb2 demonstrated expression in Sertoli cells at E14 and P0, along with selected expression in interstitial cells. TGFb3 was highly expressed at the junction of the mesonephros and gonad, in peritubular cells around the cords and in the gonocytes after birth. As shown in the current study, TGFb2 and TGFb3 expression is more predominant in the embryonic testis, while TGFb1 is more highly expressed postnatally [9], Table 1. All cells are anticipated to respond to TGFbs and the TGFb receptor type II was predominant in the whole gonad. The administration of TGFb1 to organ cultures of embryonic testis was found to reduce cell growth and tissue size, correlating to the growth inhibitory actions of TGFbs [9]. The current study used microarray analysis to confirm the previous expression analysis. TGFb2 and TGFb3 were predominant in both the embryonic

testis and ovary, with negligible levels of TGFb1. Previous observations support the presence of TGFb1 expression [9], but levels are at the limit of detection for the microarray analysis. Therefore, as previously documented TGFb isoforms are expressed in the developing gonads and the sites of action are likely at most cell types within the gonad.

Analysis of the null-mutant (-/-) TGFb knockouts demonstrated no major effect on testis histology, Figure 2, or ovarian histology, Figure 9 and (data not shown). Therefore, no gross morphological alterations on gonad development were observed. Investigation of the number of cords that develop in the E15 testis demonstrated a reduced number of cords in the TGFb2 (+/-) and (-/-) knockout animals, but no effect in the TGFb1 or TGFb3 animals, Figure 3. No effect on cord formation was observed in the P0 testis suggesting the effect in TGFb2 knockout at E15 was transient and rescued by P0 of development. Analysis of the area of the testis cords demonstrated no effect in any of the TGFb isoform knockouts. The primary morphological effect on embryonic testis development observed was a transient decrease in cord numbers in the TGFb2 knockout at E15. The TGFb1 and TGFb3 knockouts did not appear to effect testis morphogenesis. None of the TGFb isoform knockouts had an ovarian phenotype at either E15 or P0 (data not shown). The alterations in the TGFb2 knockout observed could be due to changes in cell populations, particularly the germ cell numbers. Therefore, the germ cell numbers and cellular apoptosis were investigated.

The germ cell number was examined with the GCNA immunohistochemical stain in the TGFb null-mutant animals. Neither the TGFb2 nor TGFb3 knockouts showed any effect on germ cell numbers in the testis. The TGFb1 E15 heterozygote (+/-) animals had an increase in germ cell number in the testis, while the P0 TGFb1 null-mutant (-/-) had a decrease in germ cell numbers. This inverse relationship between E15 and P0 in the TGFb1 knockouts likely reflects different functions of TGFb1 at these different developmental periods. Analysis of apoptosis with TUNEL stain was used to complement the germ cell number analysis. Interestingly, the cellular apoptosis observed in the testis was primarily in the interstitial space around and between cords, Figure 7, with no major staining in germ cells. The number of these apoptotic cells in the interstitial space was increased at E15 in the TGFb2 (+/-) and P0 TGFb3 (+/-) heterozygote animals, but no effects were observed on apoptosis in the TGFb (-/-) null-mutant animals. Therefore, no major effect on cellular apoptosis or germ cell numbers was observed. The reduction in cord formation in the TGFb2 knockout could be related to the increase in apoptosis in the interstitium observed. At E15 if the migrating mesonephros cells were reduced due to increased apoptosis, then cord formation may be reduced. Cord formation is dependent on mesonephros cell migration. Given enough time, the migrating cells may accumulate to eventually promote a similar number of cords reflected by a lack of effect observed at P0. As shown in Figure 7, the apoptotic cells observed in the TGFb2 knockout testis often associated with the cells that surround the cords. These are the cells that migrate from the mesonephros and become peritubular cells. Therefore, the observed reduction in these cells in the E15 testis could relate to the reduced cord formation observed in the TGFb2 null-mutant animals.

The embryonic ovary was found to have no major morphological alterations or effects on germ cell numbers or apoptosis in the TGFb1, TGFb2 or TGFb3 null-mutant animals at E15. At birth, the P0 germ cell numbers were found to be increased in the TGFb2 (-/-) knockout ovaries, but no effect was observed in either the TGFb1 or TGFb3 knockouts. Morphologically no major effects were seen in the ovaries, Figure 9. Since TGFb2 can influence apoptosis, the alteration in germ cell numbers could be due to an effect on the high rate of germ cell apoptosis observed in the oocyte at birth. This is associated with the assembly of the primordial follicle [3]. As seen in the testis, the primary phenotype observed was with the TGFb2 null-mutant gonadal development.

Although different TGF β isoforms have different localization, regulation and developmental control, all the TGF β isoforms cross-react with the different TGF β receptor types and can have similar functions in regards to cell growth and differentiation. Therefore, the TGF β 1, TGF β 2 and TGF β 3 can compensate for each other. The lack of major phenotypes observed is speculated to in part be due to this compensation in the individual isoform null-mutant animals. Analysis of TGF β isoform expression in the null mutant P0 testis demonstrated the alternate isoforms are expressed, with no major change in expression. Therefore, the alternate isoforms are present to compensate in the various knockouts, but direct compensation experiments remain to be performed. Unfortunately, the double knockouts die early in embryonic development prior to gonadal development. The effects observed suggest unique roles for the different TGF β isoforms during gonadal development. The TGF β 2 phenotype supports a potential role in mesonephros cell migration and cord formation in the testis, but requires further investigation.

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A.

B.

Figure 1.

Sex ratios (A) and percentages of genotypes (B) from TGFB1, TGFB2 and TGFB3 litters. Total litters in analysis for TGFB1 n=40, TGFB2 n=38, and TGFB3 n=26. In (B) open bar represents wild type (+/+), black bar represents heterozygous (+/-) and hashed bar represents homozygote knockouts (-/-). Data represents mean $\pm$ SEM.

Figure 2. Morphology of TGFb2 E15 (A-C) and TGFb1 P0 (D-F) testes cross sections from (+/+) (A, D), (+/-) (B, E) and (-/-) (C, F) animals. An H&E stained micrograph at 400X magnification is presented and representative of a minimum of 3 different animals examined.

Figure 3.

Number of seminiferous cords per mm² testis area for E15 embryos (A) and P0 postnatal pups (B) from TGFB1, TGFB2 and TGFB3 animals. Total number of embryos for E15 testis in the analysis are TGFB1 (+/+) n=3, (+/-) n=4, (-/-) n=5; TGFB2 (+/+) n=3, (+/-) n=6, (-/-) n=3; TGFB3 (+/+) n=3, (+/-) n=5, (-/-) n=6. Total number of P0 animals in the analysis are TGFB1 (+/+) n=5, (+/-) n=4, (-/-) n=8; TGFB2 (+/+) n=3, (+/-) n=5, (-/-) n=2; TGFB3 (+/+) n=4, (+/-) n=4, (-/-) n=3. Open bar represents wild type (+/+), black bar represents heterozygous (+/-) and hashed bar represents homozygote knockouts (-/-). An (a) represents statistically significant value from wildtype (+/+). Data presented as mean +/- SEM.

A.

B.

Figure 4.

Average cross sectional area of seminiferous cords per mm^2 testis area from E15 embryos (A) and P0 postnatal pups (B) from (+/+), (+/-) and (-/-) TGFB1, TGFB2, and TGFB3 animals. Total number of embryos for E15 testis in the analysis are TGFB1 (+/+) n=3, (+/-) n=4, (-/-) n=5; TGFB2 (+/+) n=3, (+/-) n=6, (-/-) n=2; TGFB3 (+/+) n=3, (+/-) n=5, (-/-) n=6. Total number of pups in the P0 testis analysis are TGFB1 (+/+) n=5, (+/-) n=3, (-/-) n=8; TGFB2 (+/+) n=3, (+/-) n=5, (-/-) n=2; TGFB3 (+/+) n=4, (+/-) n=4, (-/-) n=3. Open bar represents wild type (+/+), black bar represents heterozygous (+/-) and hashed bar represents homozygote knockouts (-/-). Data represents mean $\pm$ SEM.

Figure 5.

GCNA immunohistochemistry for TGFb1 E15 (A-C) and TGFb1 P0 (D-F) testis cross-section from (+/+) (A, D), (+/-) (B, E) and (-/-) (C, F) animals. Representative micrographs are presented from 3 animals different animals examined.

A.

B.

Figure 6.

Number of GCNA stained cells per mm² testis area for E15 (A) and P0 (B) testis from TGFB1, TGFB2 and TGFB3 animals. Total number of embryos for E15 testis in the analysis are TGFB1 (+/+) n=4, (+/-) n=4, (-/-) n=3; TGFB2 (+/+) n=3, (+/-) n=5, (-/-) n=3; TGFB3 (+/+) n=4, (+/-) n=4, (-/-) n=4. Total number of P0 animals in the analysis are TGFB1 (+/+) n=3, (+/-) n=4, (-/-) n=4; TGFB2 (+/+) n=4, (+/-) n=3, (-/-) n=2; TGFB3 (+/+) n=5, (+/-) n=4, (-/-) n=3. Open bar represents wild type (+/+), black bar represents heterozygous (+/-) and hashed bar represents homozygote knockouts (-/-). An (a) represents statistically significant value from wildtype (+/+). Data represents mean $\pm$ SEM.

Figure 7. Apoptosis TUNEL stained TGFb2 E15 (A-B) and TGFb2 P0 (C-D) testis cross-section from wildtype (+/+) (A, C) and homozygote (-/-) (B, D) mice. Representative micrograph with apoptotic cells (yellow) at 400X magnification.

A.

B.

Figure 8.

Number of TUNEL labeled cells per mm² testis area for E15 (A) and P0 (B) TGFB1, TGFB2 and TGFB3 animals. Total number of embryos in the analysis are TGFB1 (+/+) n=5, (+/-) n=3, (-/-) n=6; TGFB2 (+/+) n=3, (+/-) n=10, (-/-) n=3; TGFB3 (+/+) n=5, (+/-) n=5, (-/-) n=5. Total number of P0 animals in the analysis are TGFB1 (+/+) n=3, (+/-) n=3, (-/-) n=5; TGFB2 (+/+) n=6, (+/-) n=5, (-/-) n=2; TGFB3 (+/+) n=3, (+/-) n=3, (-/-) n=3. Open bar represents wild type (+/+), black bar represents heterozygous (+/-) and hashed bar represents homozygote knockouts (-/-). An (a) represents statistically significant value from wildtype (+/+). Data represents mean +/- SEM.

Figure 9.

Ovary micrograph of H&E (A,B) and GCNA (C,D) ovarian cross-sections from TGFB2 P0 (+/+) (A, C) and (-/-) (B, D) animals. (E) Number of germ cells per mm² of ovarian cross section for P0 pups from TGFB1, TGFB2, and TGFB3 animals. Total number of animals in the analysis are TGFB1 (+/+) and (+/-) n=7, (-/-) n=4; TGFB2 (+/+) and (+/-) n=5, (-/-) n=2; TGFB3 (+/+) and (+/-) n=5, (-/-) n=3. Open bar represents combined wild type (+/+) and heterozygous (+/-) and black bar represents homozygote knockouts (-/-). Data represents mean $\pm$ SEM.

TABLE 1

A. Microarray expression levels (mean raw signal) of Transforming Growth Factor (TGF) β 1, β 2, β 3 and receptors I, II and III during rat gonadal development.					
Testis	E13	E14	E16	P0	P6
TGF β 1	A	A	A	A	A
TGF β 2	70.2	69.1	101.2	119.5	65.0
TGF β 3	36.9	59.4	120.2	85.0	95.4
β -receptor I	53.6	59.6	50.6	A	46.2
β -receptor II	203.9	213.5	240.2	233.2	726.8
β -receptor III	86.6	97.9	102.3	369.1	92.9
Ovary					
TGF β 1	A	A	A	ND	ND
TGF β 2	76.1	77.0	80.0	ND	ND
TGF β 3	41.2	59.4	70.5	ND	ND
β -receptor I	34.0	43.2	A	ND	ND
β -receptor II	190.3	193.7	215.0	ND	ND
β -receptor III	95.9	90.8	77.9	ND	ND
B. Microarray expression levels (mean raw signal) of Transforming Growth Factor (TGF) β 1, β 2, β 3 and receptors I, II and III during mouse gonadal development.					
Testis	E11.5	E12.5	E16.5	P0	P6
TGF β 1	A	A	A	A	A
TGF β 2	42.4	64.1	69.6	59.7	36.5
TGF β 3	83.7	124.3	158.3	184.6	196.3
β -receptor I	32.7	40.3	A	15.9	26.3
β -receptor II	A	A	A	A	A
β -receptor III	A	A	51.1	68.5	90.1
Ovary					
TGF β 1	A	A	A	ND	ND
TGF β 2	51.4	48.4	50.1	ND	ND
TGF β 3	92.4	105.3	175.8	ND	ND
β -receptor I	A	48.4	A	ND	ND
β -receptor II	A	A	A	ND	ND
β -receptor III	41.2	61.7	50.5	ND	ND

Statistically significant present call microarray signals are presented with E = embryonic day (E0 = post-natal day. "A" denotes transcript was not detected by the array analyses. "ND" denotes samples not analyzed.