Smad4 Overexpression Causes Germ Cell Ablation and Leydig Cell Hyperplasia in Transgenic Mice

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Members of the transforming growth factor- β (TGF- β) superfamily play a variety of important roles in testicular development and function. The tumor suppressor gene, *Smad4*, is a common mediator of TGF- β , activin, and bone morphogenetic protein-mediated signaling pathways. To investigate the role of the *Smad4* gene during testicular development and function, transgenic mice were generated using a Flag-tagged *Smad4* gene driven by 180-bp fragment of the Mullerian inhibiting substance upstream promoter sequence. Three *Smad4* transgenic founders (A, B, and G) were detected by Southern blot analysis; line B showed the highest expression of the *Smad4* transgene and was further studied. The fertility in F1 generation (B) and F2 generation (BB) of the *Smad4* transgenic mice was not impaired. However, in the F3 generation (B2x) all animals were impacted by the overexpression of the *Smad4* transgene and two kinds of phenotypes were observed. In one group animals were completely infertile, while in the other group animals were fertile and sired the normal number of pups/litter. These groups are designated as infertile and fertile in the text. Histological evaluation of the testes from the infertile group showed variable degrees of Leydig cell hyperplasia, apoptosis of germ cells, spermatogenic arrest, seminiferous tubule degeneration, and infertility. In the fertile group, there was no apparent change in the histology of the testis except for a slight increase in the number of Leydig cells. Serum follicle-stimulating hormone levels in the adult animals of both groups of *Smad4* transgenic male mice were not significantly different from normal littermates; however, testosterone levels in both groups were significantly ($P < 0.05$) increased. These results suggest that overexpression of *Smad4* leads to testicular abnormalities and infertility supporting the hypothesis that the TGF- β signaling pathways are carefully orchestrated during testicular development. In the absence of normal levels of *Smad4* testicular function is compromised. (*Am J Pathol* 2002, 161:1723–1734)

Testicular development and cellular functions are under the control of hormones, growth factors, and cytokines.^{1–3} Among the growth factors essential to testis function are various peptide families, including the fibroblast growth factors, epidermal growth factors, and transforming growth factor- β s (TGF- β s). In mammals, the TGF- β superfamily contains a growing number of structurally related but functionally different polypeptides, including TGF- β 1, TGF- β 2, TGF- β 3, activins, inhibins, Mullerian inhibiting substance (MIS), and bone morphogenetic proteins (BMPs).^{4–6} These polypeptides elicit a wide range of biological effects on cell proliferation, survival, differentiation, bone formation, regulation of hormone secretion, and various other developmental functions.^{7–10} In the testis, some of these TGF- β -related peptides, for example, inhibins, activins, and BMPs, have been reported to affect testicular function including maintenance of spermatogenesis and Leydig cell steroidogenesis.^{11–15} Furthermore, recent observations in transgenic and knockout mice in which genes related to the TGF- β peptide families were manipulated indicated that the reproductive function in these animals was affected.^{14,16,17} From these studies it seems that the action of the TGF- β superfamily is of major importance to the testis in terms of both development and function (ie, steroidogenesis and gametogenesis).

Paradoxically, studies regarding the testicular activity of TGF- β ligands and related peptides have revealed very little information about their signaling pathways in this tissue. Members of the TGF- β superfamily transduce signals through two different types of serine/threonine protein kinase receptors, known as type I and type II receptors.¹⁸ On ligand binding, the type II receptor transphosphorylates and activates the type I receptor, which then activates the downstream signal transduction cascade. Recent studies have shown that *Smad* proteins, first identified through genetic screens in *Drosophila* and *Caenorhabditis elegans*, play pivotal roles in transducing signals from TGF- β ligands.^{19–21} On the basis of structural and functional criteria, the *Smad* family can be di-

Supported by the National Institutes of Health (grants HD 35708 and HD 05863).

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Accepted for publication August 1, 2002.

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vided into three subgroups. The receptor-regulated *Smads* (*Smad1*, 2, 3, 5, and 8), the common *Smad* (*Smad4*/DPC4), and the inhibitory *Smads* (*Smad6* and 7). *Smad1*, 5, and 8 mediate BMP-signaling pathways,^{20,22–24} whereas *Smad2* and *Smad3* have been shown to transduce activin/TGF- β -signaling pathways.^{25,26} After activation by their respective type I receptor kinases, the receptor-specific *Smads* appear to complex with the common-partner, *Smad4*.²⁷ Then the receptor-regulated *Smad*/*Smad4* complexes translocate to the nucleus where they participate in the activation of target gene transcription.²⁸ *Smad6* and *Smad7* function as antagonists in the signaling process by forming a stable interaction with type I receptors and blocking the activation of receptor-regulated *Smad* or interfering with the formation of receptor-regulated *Smad*/*Smad4* complexes.²⁹ The discovery of *Smad* proteins has clearly advanced our understanding regarding TGF- β signaling from its cognate receptor to the nucleus.

The information available on the distribution and function of *Smad* proteins in the testis is extremely limited. Spermatogenesis is a unique and complex developmental process. Spermatogonia, the stem cell population, undergo mitosis and differentiate into primary spermatocytes. These cells in turn undergo meiosis, and differentiate into secondary spermatocytes, spermatids, and spermatozoa. *Smad1* is expressed in pachytene spermatocytes to stage 1 spermatids.³⁰ *Smad2* mRNA and protein have been detected in meiotic germ cells, interstitial cells, and Sertoli cells of the testis.³¹ *Smad7* is selectively expressed in the seminiferous tubules.³² The mRNAs for *Smads* 1 to 7 have been identified in cultured Leydig cells, indicating the presence of a functional pathway in these cells.³³ *Smad4* is expressed in a ubiquitous manner consistent with the fact that this protein is essential to all TGF- β superfamily ligand signaling.³²

Targeted mutations of *Smad* genes have revealed specific developmental and physiological functions of cytoplasmic co-activators. *Smad2*-deficient mice died as early as embryonic day 6.5 because of failure in egg cylinder elongation, mesoderm formation, and gastrulation.^{34–36} In contrast, *Smad3*-deficient mice were born alive but died between 1 to 7 months of age because of impaired immunity and metastatic colorectal cancer.^{37,38} *Smad4*-deficient mice died shortly after implantation because of defects in primitive endoderm formation.^{39,40} Because *Smad4* is a common mediator *Smad* for TGF- β , activin, and BMP mediated-signaling and is absolutely necessary for development and viability, a study was designed in which *Smad4* expression was altered in a tissue-specific manner. The aim of the present study was to investigate the functional role of *Smad4* in the male mice. Therefore, transgenic mice using a FLAG-tagged *Smad4* gene driven by a 180-bp fragment of the MIS upstream promoter sequence were generated. Overexpression of *Smad4* in male mice caused a primary testicular defect leading to infertility. These results suggest that the level of *Smad4* expression is critical for TGF- β -mediated signaling pathways that are involved in the development and function of the normal testis.

Materials and Methods

Generation of pMIS-*Smad4* Transgenic Mice

The transgenic cassette was generated by cloning a fragment of the MIS upstream promoter (–200 to + 8) that contains the region (–180 to + 1), previously validated to be sufficient for expression of human growth hormone⁴¹ and human DAX-1⁴² in Sertoli cells. This promoter fragment was cloned upstream of *Smad4* cDNA (provided by Jeff Wrana, Hospital for Sick Children, University of Toronto, Toronto, Canada). The *Smad4* construct was inserted between the Flag epitope (DYKD-DDDK) and a human growth hormone polyadenylation sequence at the 3' end. The transgenic founders were generated at the Northwestern University Transgenic and Targeted Mutagenesis Facilities (CMIER) under the supervision of Dr. Lynn Doglio. Approximately 2 ng/ μ l of linearized (*EcoRI*) DNA of the transgenic construct was injected into the pronucleus of one-cell stage mouse embryos obtained from CD1 females (Harlan Sprague Dawley, Indianapolis, IN). The embryos were transferred to pseudopregnant foster mothers. Genomic DNA was extracted (Promega Wizard Genomic DNA Purification System; Promega, Madison, WI) from tail biopsies and the *Smad4* transgenic founders were distinguished by Southern blot analysis. Three separate lines (A, B, and G) were detected by Southern blot analysis; one line was studied further. The transgenic animals in subsequent generations were determined by polymerase chain reaction of genomic DNA. The primers used corresponded to the *Smad4*-coding region (5'-ACC ATG GAC TAC AAG GAC GAC-3') and the Flag epitope (5'-TGGGG TGC TGA AGA TGG CCG TT-3'). Amplification of the transgene was performed for 30 cycles using an annealing temperature of 58°C. Dot blot analyses using 3 μ g of genomic DNA were performed to confirm the presence of transgene.

Breeding of Transgenic Mice

All mice were housed and bred in the Northwestern University Center for Comparative Medicine barrier facility with controlled photoperiods (14 hours light and 10 hours darkness), temperature, and humidity. The animals were maintained and treated in accordance with the policies of Northwestern University's Animal Care and Use Committee. Founder mice from the B line of *Smad4* transgenic mice were crossed with CD1 female mice to generate a heterozygous F1 generation. Additional mice were generated by inbreeding the respective generations of transgenic mice. Hence, the BB line resulted from inbreeding the F1 (B) generation and the B2x line resulted from inbreeding the F2 (BB) generation. To assess fertility, selective breeding pairs of all transgenic lines and wild-type CD1 mice were housed in individual cages and the average numbers of pups per litter produced throughout a period of 3 to 5 months were recorded. Fecundity was assessed based on the number of pups per litter weaned at 21 days.

Western Blot Analysis

Neonatal testes from transgenic (B2x) and normal littermate (NLM) mice were collected and immediately frozen on dry ice. The frozen testes from each group were pooled and ground by mortar and pestles. The ground tissue was collected and extracted in 60 μ l of fresh protein extraction solution (10 mmol/L Tris, pH 7.6, 0.5 mol/L NaCl, 1 mol/L $MgCl_2$, 0.1% Triton X-100, and one tablet of BMPI (protease inhibitor) per 10 ml of solution. The extracted tissue was exposed to two 10-minute rounds of freeze-thaw cycles (dry ice and 37°C), briefly centrifuged, and the supernatant was transferred to a new tube. Protein concentration was determined by the Bradford assay, and an equal amount (90 μ g) of protein from both NLM and *Smad4* transgenic groups were loaded onto the gel (NuPAGE 4 to 12% Bis-Tris gel) for electrophoresis. Before loading, the protein was mixed with 4 $\times$ NuPAGE LDS (Novex, Carlsbad, CA) at a concentration of 0.1 mol/L of dithiothreitol and heated for 10 minutes at 70°C. The gel was immersed in 1 $\times$ MES running buffer (50 mmol/L 2-(*n*-morpholino)-ethanesulfonic acid, 50 mmol/L Tris base, 3.5 mmol/L sodium dodecyl sulfate, 1 mmol/L ethylenediaminetetraacetic acid) to which 200 μ l of NuPAGE antioxidant was added to the inner chamber of the gel apparatus according to the manufacturer's instructions. After electrophoreses, the proteins in the gel were transferred to a polyvinylidene difluoride membrane under electrical gradient for 1 hour in a transfer apparatus. The blot was incubated overnight at 4°C shaking in 5% milk-Tris-buffered saline (TBS)-Tween blocking solution to which goat IgG (Sigma Chemical Co., St. Louis, MO) at a concentration of 1 μ g/ μ l was added. Primary antibody, monoclonal mouse anti-Flag (Sigma Chemical Co.), was applied for 1 hour the next morning at a final concentration of 1 μ g/ μ l in 2.5% milk-TBS-Tween solution. After three washings in TBS-Tween, the blot was incubated for 3 hours with a secondary antibody, goat anti-mouse IgG, conjugated to horseradish peroxidase (Bio-Rad, Hercules, CA) at a concentration of 10 μ l/ml. The antigen-antibody binding was detected with an ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ) using 25 minutes of exposure to Hyperfilm MP (Amersham Pharmacia Biotech).

Histology

Testes from 2-day (neonatal), 21-day (3 weeks), and 45 (6 weeks)-day-old NLMs and *Smad4* transgenic mice were rapidly dissected and fixed in Bouin's solution at 4°C for 2 to 24 hours (the time depending on the size of the testis). Dehydration of tissues was done in a series of ascending concentrations of ethanol for a period of 2 to 5 hours. The tissues were embedded in paraffin and 5- μ m sections were cut. Sections were stained with hematoxylin and eosin.

Immunohistochemistry

Immunohistochemistry was performed according to the procedure described by Kroft and colleagues.⁴³ Briefly,

sections were deparaffinized by incubating twice for 5 minutes each time in xylene and then rehydrated by incubating twice, for each time, 3 minutes in 100% (v/v) ethanol, 3 minutes in 95% ethanol, 20 minutes in 70% ethanol containing 1% (w/v) H_2O_2 (to inactivate endogenous peroxidase activity), 20 minutes in 70% ethanol saturated with Li_2CO_3 (to neutralize picric acid from the fixative), 3 minutes in 50% ethanol, 10 minutes in dd H_2O , 10 minutes in phosphate-buffered saline (PBS) (pH 7.2), and 5 minutes in 300 mmol/L of glycine in PBS. The remainder of the protocol was performed using reagents from the Zymed Histostain-plus kit (Zymed Laboratories, Inc., South San Francisco, CA). All operations were performed at room temperature unless otherwise stated. Tissue sections were circled with a PAP Pen (Research Products International Corp., Mount Prospect, IL) to provide a well for reagents. Sections were blocked for 10 minutes with blocking solution and then incubated overnight in a humidified chamber at 4°C with primary anti-serum diluted in PBS containing 3% (w/v) bovine serum albumin and 0.1% (v/v) Triton X-100. The primary antibodies used were rabbit anti-mouse LDH-C4 antibody (1:3000 dilution), rabbit anti- β A (305-24-D, amino acids 81 to 113, dilution 1:100), β B (305-25-D, amino acids 80 to 1120, dilution 1:75)-subunit polyclonal antibodies,⁴⁴ mouse follistatin monoclonal antibody (1:1000 dilution), and a rabbit anti-mouse STAR antibody (1:1000 dilution; kindly provided by Dr DM Stocco, Texas Tech University, Lubbock, TX). Sections were washed twice for 5 minutes each time and once for 10 minutes in PBS. Sections were incubated for 30 minutes with secondary antiserum and then washed twice for 5 minutes each time and once for 10 minutes in PBS as above. Sections were incubated for 10 minutes with enzyme conjugate, followed by two 5-minute washes and one 10-minute wash in PBS. Staining was visualized by incubation with 3,3'-diaminobenzidine (brown color) or AEC chromogen (red color) for 1 to 3 minutes. Slides were then rinsed well with dd H_2O , counterstained with hematoxylin, and dehydrated by incubation for 3 minutes in 50% ethanol, 3 minutes in 70% ethanol, 3 minutes in 95% ethanol, and twice for 3 minutes each time in 100% ethanol. Sections were incubated for 3 minutes in xylene, air-dried, and mounted with Cytoseal (VWR Scientific, McGaw Park, IL).

In Situ Detection of Apoptosis

The ApopTag method for *in situ* apoptosis detection of programmed cell death was performed using testes from 6-week-old wild-type and *Smad4* transgenic mice. The TUNEL (terminal dUTP nick-end labeling) method uses the terminal deoxynucleotidyl transferase (TdT) enzyme to catalyze the attachment of digoxigenin-dUTP onto the free 3'-OH ends of DNA fragments (Intergen Co., NY). Paraffin-embedded testes sections were rehydrated in a graded series of alcohol. After blocking with 1% bovine serum albumin-PBS, tissue sections were incubated for 1 hour at 37°C with digoxigenin-dUTP and TdT or with 1% PBS as the control. Tissue sections were then incubated in anti-digoxigenin fluorescein conjugate for 30 minutes

at room temperature. The antigen-antibody complex was visualized by fluorescence microscopy.

Serum Hormone Measurements

Serum testosterone and follicle-stimulating hormone (FSH) levels in 6-week-old wild-type and *Smad4* transgenic mice were measured by RIA at the Northwestern University P30 Center RIA Core Facility under the direction of Dr. Jon Levine. National Institute of Diabetes and Digestive and Kidney Diseases antiserum and standards (rFSH-RP-2 standard/rFSH-S-11 antibody) were used for FSH measurements. The results are expressed as ng/ml. FSH assay sensitivity was 0.05 ng/sample or 1 ng/ml. A testosterone double-antibody RIA kit (ICN, Costa Mesa, CA) containing antibodies and standards was used for RIA. The sensitivity of the testosterone assay was 2 pg/ml.

Statistical Analysis

All values are expressed as the mean $\pm$ SEM. Student's *t*-test was used to evaluate differences between NLMs and 6-week-old *Smad4* transgenic mice sera. $P < 0.05$ was considered statistically significant.

Results

Expression and Analysis of the *Smad4* Transgene

To study the effect of *Smad4* overexpression in transgenic mice, cDNA coding for the *Smad4* gene was expressed under the control of a MIS promoter (Figure 1A). Three separate lines (A, B, and G) were detected by Southern blot analysis; line B showed the highest expression of the *Smad4* transgene, therefore, this line was further studied (Figure 1B). The *Smad4* transgenic mice from the B, BB, and B2x lines appeared healthy and did not exhibit any macroscopic physical aberrations or reduction in body weight. To confirm expression of *Smad4* transgene, the Flag-epitope tag was detected by Western blot analysis using testis extracts from neonatal mice (Figure 1C). As predicted by the genotype, the Flag protein was detected in the testes from transgenic animals at a molecular weight appropriate for *Smad4* and not in the age-matched wild-type littermates. The Flag epitope could not be immunolocalized in testicular sections from *Smad4* neonatal mice. This may be because of the conformation of the native epitope in the context of the *Smad4* protein. Regardless, the Flag antibody is not ideal because it recognizes a variety of nonspecific proteins, as suggested by the Western blot analysis (Figure 1C).

Fertility Studies

Continuous mating studies were performed to assess fertility in NLMs and *Smad4* transgenic mice. Twelve-week-old males were housed with 8-week-old CD1 wild-type female mice and the fertility and number of litters produced by each pair was measured. The fertility of

Figure 1. The generation of *Smad4* transgenic mice. **A:** A schematic of the Flag-*Smad4* transgene. A 200-bp MIS promoter fragment was fused to a Flag-*Smad4* expression construct. Polymerase chain reaction primers were used to amplify a probe used in Southern blot analysis (arrows). Restriction sites were used to linearize or excise the insert and prepare genomic DNA for Southern blot analysis. **B:** Southern blot analysis of transgenic genomic DNA. Transgenic mice were identified by Southern blot analysis using the 1030-bp *Smad4*-coding region fragment as probe. Three founders were identified (lines A, B, and G). **Lane B** is the mouse founder used in these studies. **C:** Flag transgene expression in mouse testicular tissue. Total protein was extracted from testes of neonatal *Smad4* transgenic mice and NLMs. Equivalent protein was loaded and subjected to Western blot analysis. The anti-Flag antibody recognizes a variety of nonspecific proteins but only one protein in the *Smad4*-Flag lane at the predicted molecular size for the *Smad4*-Flag protein was observed.

male mice in B as well as BB lines was not different from NLMs; the number of pups per litter in these lines were 11.75 ± 0.85 , 9.6 ± 0.75 , and 12.44 ± 0.80 in B, BB, and NLMs, respectively. However, in the B2x line 5 of 11 animals were infertile while 6 reproduced normally with the average of 9.5 ± 1 pups per litter ($P = \text{NS}$). Based on differences in the fertility status of animals of the B2x transgenic line, animals were classified as one of the two groups: animals that had complete spermatogenic arrest and sterile were categorized as the infertile group, and animals that were fertile and sired a normal number of pups per litter were categorized as the fertile group. All results in the infertile and fertile groups of *Smad4* transgenic mice were compared with age-matched NLMs.

Morphological and Histological Analyses

To determine the cause(s) of infertility in the *Smad4* transgenic mice, morphological and histological analyses were performed. There was no change in the body weight of animals (6 weeks old) of the B, BB, and B2x lines (both infertile and fertile groups) when compared to NLMs (data not shown). The mean testes size in animals of B, BB, and B2x lines before 6 weeks of age were not different (data not shown). The average testes weight of adult wild-type controls was 229.6 ± 11.23 mg ($n = 8$). In the BB line, 2 of 12 animals showed a significant decrease in the testes size. The average testes size in these 2 animals

Table 1. Effects of Overexpression of *Smad4* on the Body Weight, Testes Volume, Fertility, and Serum Hormones in Adult (6 Weeks) Mice

Parameters	Normal littermates	Infertile group (<i>Smad4</i>)	Fertile group (<i>Smad4</i>)
Body weight (g)	36.40 ± 0.88	32.9 ± 0.29	32.9 ± 0.71
Testes weight (mg)	229.6 ± 11.23	81.2 ± 19.7*	145.03 ± 22.7*
No. of pups/litter	12.4 ± 0.80	0	11.2 ± 0.89
Testosterone (ng/mL)	1.39 ± 0.76	3.31 ± 0.42*	6.41 ± 1.91*
FSH (ng/mL)	35.28 ± 2.06	39.11 ± 1.63	39.58 ± 1.27

* $P < 0.05$; significant difference in relation to normal littermates.

were 100 ± 0.01 mg ($P < 0.05$), whereas in the remaining 10 animals the average testes weight was 230 ± 0.01 mg ($P = \text{NS}$). In the B2x line, testes size in all animals was significantly reduced as compared to NLMs. The mean testes size was 81.2 ± 19.7 mg ($P < 0.05$) and 145.03 ± 22.7 mg ($P < 0.05$), in the infertile and fertile groups, respectively (Table 1).

The histology of testes from animals of B and BB line did not show any change from NLMs at any point of testicular development (data not shown). Because animals of the B2x line had small testes as compared to NLMs, the histological changes were studied in detail for the animals of the B2x line only. Neonatal animals of both groups (infertile and fertile) of the B2x line contained gonocytes and Sertoli cells, and appeared normal as compared to NLMs (data not shown). However, testes from 3- and 6-week-old mice in the infertile group and not in the fertile group of *Smad4* transgenic mice showed abnormal spermatogenesis. Figure 2A shows testis section from a NLMs at 3 weeks of age. The most advanced germ cells in seminiferous tubules were pachytene and diplotene spermatocytes, indicating a first wave of spermatogenesis. Figure 2B shows histology of testis from a 3-week-old mouse from the infertile group. As can be seen, there is a marked decrease in seminiferous tubule diameter and in the number of primary spermatocytes. In general, spermatogenesis appeared to be completely arrested. Figure 2C shows a testis section from 3-week-old mice from the fertile group of *Smad4* transgenic mice. The tubule structure appeared normal showing the presence of Sertoli cells, spermatogonia, and spermatocytes. Testis from 6-week-old NLMs (Figure 2D) revealed closely packed seminiferous tubules with clear evidence of all stages of spermatogenesis and spermiogenesis, including spermatogonia in the basal layer of each tubule, and round and elongated spermatids toward the lumen. In contrast, testes of *Smad4* transgenic mice from the infertile group at 6 weeks of age showed complete spermatogenic arrest (Figure 2E). Most of the seminiferous tubules contained Sertoli cells and a few spermatogonia and there was a complete absence of developing spermatids and spermatozoa. Numerous degenerating cells were observed in those atrophic tubules. In addition, these animals also showed variable degrees of Leydig cell hyperplasia (Figure 2E). As might be expected and consistent with their fertility, testes of animals from the fertile group showed normal stages of spermatogenesis, however an increase in the number of Leydig cells was observed (Figure 2F). The seminiferous tubular di-

ameter appeared smaller than NLMs, and this was consistent with 50% reduction in testis size in this subgroup.

To confirm the stage at which spermatogenic arrest occurred, various testis-specific proteins were immunolocalized in the testis of 3- and 6-week-old NLMs and *Smad4* transgenic mice. We used LDH-C₄ protein as a specific marker for germ cells. LDH-C₄ is a testis-specific protein that is normally found in the cytoplasm of germinal epithelial cells from pachytene spermatocytes to elongated spermatids.⁴⁵ Figure 3A shows LDH-C₄ localization in the spermatocytes of 3-week-old NLMs. Figure 3B shows a testis section from a NLM incubated with normal mouse serum instead of primary antibody, showing absence of immunostaining reaction in germ cells. Figure 3, C and D, shows testis sections from the infertile group of *Smad4* transgenic mice where pachytene spermatocytes stained with LDH-C₄ antiserum (in brown color) started to slough off from the Sertoli cell cytoplasm at 3 weeks of age, indicating germ cell maturation arrest beyond pachytene spermatocyte stage (Figure 3, C and D). Additionally, most *Smad4* transgenic mice showed matrix deposition and a massive increase in the number of cells in the interstitial spaces. To confirm whether actively dividing cells between interstitial spaces are Leydig cells, we used antibodies against a Leydig cell-specific marker, steroidogenic acute regulatory (STAR) protein. STAR protein is associated with acute regulation of Leydig cell testosterone biosynthesis by luteinizing hormone.⁴⁶ Figure 4A shows a testis section from a NLM depicting the presence of a normal number of Leydig cells that show localization of STAR protein. Figure 4B shows a preimmune serum control in which no immunostaining for STAR protein was detected in Leydig cells. Figure 4, C and D, shows massive Leydig cell hyperplasia in the infertile group of *Smad4* transgenic mice. The Leydig cell hyperplasia that we observed is similar to the findings in follistatin transgenic,⁴⁷ MIS knockout,⁴⁸ or MIS/inhibin- α double-knockout mice.⁴⁹ To determine whether follistatin was up-regulated, follistatin was localized using testis sections from 6-week-old *Smad4* transgenic mice from the infertile group. Figure 5, A and B, shows follistatin localization in Sertoli and Leydig cells. The level of follistatin appeared to be unaltered on a per cell basis between NLMs (Figure 5A) and the *Smad4* transgenic mice (Figure 5B). Figure 5C is a preimmune serum control in which no signal for follistatin was detected in Sertoli and Leydig cells.

To determine the effect of *Smad4* overexpression on activin levels in the testis, we localized activin/inhibin β A

Figure 2. Histology of testes from *Smad4* transgenic mice and NLMs stained with H&E. **A–C:** Testis sections were prepared from 3-week-old mice. **A:** A normal littermate testis section. **Arrow** indicates spermatogonia (SG), primary and secondary spermatocytes (SP), and Sertoli cells (SC). Leydig cells are indicated by an **arrowhead**. **B:** Testis section from a representative infertile group of *Smad4* transgenic mice. Few spermatogonia, Sertoli cells, and spermatocytes (indicated by **arrows**) are detected and the seminiferous tubule diameter is less than the normal littermate. **C:** Testis from the representative fertile group of *Smad4* transgenic mice showing normal progression of spermatogenesis. **D–F:** Testis sections from 6-week-old mice. **D:** Testis from a normal littermate. Closely packed seminiferous tubules [containing spermatogonia, spermatocytes, round spermatids (rST), and elongated spermatids (eST)], a limited interstitial compartment (LC), and complete spermatogenesis as supported by abundance of elongated spermatids was observed. **E:** Testis section of a representative transgenic animal from the infertile group showing clear degeneration of seminiferous epithelium with dilation of lumen. Sertoli cells and few spermatogonia are present (**arrows**). Leydig cell (LC) hyperplasia is also prevalent. **F:** Testis section from the fertile group of *Smad4* transgenic mice showing normal spermatogenesis, but an increase in the number of Leydig cells (LC). Original magnifications, $\times 400$.

Figure 3. Immunolocalization of LDH-C4 in the testis of 3-week-old NLMs and *Smad4* transgenic mice. All sections were stained with DAB (brown) and counterstained with hematoxylin (blue). **A:** Testis section from a normal littermate showing LDH-C4 expression (in brown color) in pachytene spermatocytes (SP) (arrow). Note, in spermatogonia (SG) there is no expression of LDH-C4. **B:** Testis section from a normal littermate incubated with normal mouse serum instead of primary antibody showing absence of immunostaining reaction in pachytene spermatocytes (SP). Some background staining to extracellular matrix and interstitial cells is noted with this antibody. **C:** Arrow points to sloughing of spermatocytes in the infertile group of *Smad4* transgenic mice. Spermatogonia (SG) are present near the periphery of the tubule. Original magnifications: $\times 200$ (A–C); $\times 400$ (D, a higher magnification of C).

and β B subunits in 6-week-old NLMs and in the infertile group of *Smad4* transgenic mice. We found a weak signal for β A-subunit of activin/inhibin in the testis of NLMs and *Smad4* transgenic mice (Figure 5, D and E, respectively). However, for the β B-subunit of activin/inhibin, an intense red signal was detected in Sertoli and Leydig cells of both NLMs and *Smad4* transgenic mice (Figure 5, G and H, respectively). Similar to follistatin, there was no difference in the protein levels on a per cell basis between NLMs and the *Smad4* transgenic mice. Figure 5, F and I, shows preimmune serum controls in which no signal for β A and β B was detected in Sertoli and Leydig cells.

To further investigate sperm production and maturation, cauda epididymides of NLMs and an animal from the infertile group of *Smad4* transgenic mice was examined (Figure 6). As can be observed, the columnar epithelium in *Smad4* transgenic mice did not seem to be altered in its morphological appearance compared to the NLMs (Figure 6A). However, in contrast, the lumen was virtually devoid of spermatozoa in the infertile group of *Smad4* transgenic mice (Figure 6B).

In Situ Detection of Apoptosis

The presence of numerous degenerating cells in the atrophic tubules of animals from the infertile group prompted us to determine whether these cells are apoptotic. TUNEL revealed that many seminiferous tubules in the testes from the infertile group of *Smad4* transgenic mice contained numerous spermatocytes undergoing cell death as compared to NLMs (Figure 7, A and B).

Serum Hormone Measurements

Because *Smad4* transgenic mice had smaller testes and showed a severe disruption of spermatogenesis, serum FSH and testosterone levels were measured. Table 1 shows FSH and T levels in 6-week-old NLMs and infertile and fertile groups of *Smad4* transgenic mice. In both groups of *Smad4* transgenic mice, FSH levels were not statistically different from NLMs. The FSH levels were 35.28 ± 2.06 ng/ml, 39.11 ± 1.63 ng/ml ($P = \text{NS}$), and

Figure 4. Immunolocalization of STAR in the testis sections from 6-week-old *Smad4* transgenic mice and normal littermate. Sections were stained with AEC (red) and counterstained with hematoxylin (blue). **A:** STAR protein (in red color) is detected in the Leydig cells (LC) in testis sections from a normal littermate (**arrow**). **B:** Testis section from a normal littermate incubated with normal mouse serum instead of primary antibody showing absence of immunostaining reaction in Leydig cells (LC). **C:** Testis section from the infertile group of *Smad4* transgenic mice. STAR protein is immunolocalized to hyperplastic Leydig cells (LC) (**arrow**). **D:** A higher magnification of **C**. Original magnifications: $\times 200$ (**A**, **B**, and **D**); $\times 100$ (**C**).

39.58 ± 1.2 ng/ml ($P = \text{NS}$), respectively, in the NLMs, infertile, and fertile groups of *Smad4* transgenic mice. The testosterone levels in NLMs were 1.39 ± 0.76 ng/ml. In contrast to FSH, testosterone levels were significantly increased ($P < 0.05$) in both infertile and fertile groups of *Smad4* transgenic mice as compared to age-matched NLMs. The increase in testosterone levels was greater in the fertile group, 6.41 ± 1.91 ng/ml (where spermatogenesis looked normal) as compared to 3.31 ± 0.42 ng/ml in the infertile group (with abnormal spermatogenesis and massive Leydig cell hyperplasia) (Table 1).

Discussion

Smad4 was originally identified as a candidate tumor suppressor gene, DPC4, that was somatically deleted in 50% of all human pancreatic cancers and in a subset of colorectal cancers.^{50–52} Recently, an insertional mutation was detected in the *Smad4* gene in seminoma testicular germ cell tumors in men;⁵³ however, it is not known whether this was an activating or an inactivating mutation.

In the mouse, *Smad4* gene disruption studies were conducted to characterize the functional role of *Smad4*; deletion of the *Smad4* gene resulted in embryonic lethality.^{39,40} To further understand the physiological role of *Smad4*, particularly in male reproduction, we generated gain-of-function *Smad4* transgenic mice using MIS promoter. The MIS promoter has been shown to be sufficient for the initiation and maintenance of Sertoli-cell-specific expression of a human DAX-1 gene, and is thus an appropriate promoter for studying the overexpression of this transcription factor in that compartment.⁴²

In contrast to mice deficient in the *Smad4* gene, *Smad4* transgenic mice were viable and developed to adulthood. However, *Smad4* transgenic male mice had various levels of infertility. All animals were impacted by the overexpression of the *Smad4* transgene. Interestingly, two kinds of phenotypes were observed in our colony. In one group, the overexpression of the *Smad4* transgene resulted in a primary testicular defect with progressive seminiferous tubule epithelial degeneration, leading to a severe depletion of germ cells, a reduction in testis size,

Figure 5. Immunolocalization of follistatin and activin/inhibin β A and β B subunits in the testis of 6-week-old mice. Sections were stained with AEC (red) and counterstained with hematoxylin (blue). **A:** A red signal for follistatin is apparent in Sertoli (SC) (arrowhead) and Leydig cells (LC) (arrow) of the testis from NLMs. **B:** Follistatin immunolocalization in a testis section from the infertile group of *Smad4* transgenic mice. Arrowhead and arrow point to Sertoli and Leydig cells, respectively. **C:** Preimmune serum control showing absence of immunostaining in Leydig (arrow) and Sertoli cells (arrowhead). **D and E:** Immunolocalization of β A subunit of activin/inhibin in the testis sections from a NLM and the infertile group of *Smad4* transgenic mice, respectively. A weak signal for β A was detected in Leydig cells (LC) (arrow) of both NLMs and *Smad4* transgenic mice, respectively. **F:** A preimmune control showing absence of any positive signal for β A-subunit of activin/inhibin subunit in the testis. **G and H:** Testis sections from a NLM and the infertile group of *Smad4* transgenic mice, respectively. A red signal for β B-subunit of activin/inhibin was detected in Sertoli (arrowhead) and Leydig cells (arrow), respectively. **I:** Preimmune serum control for β B-subunit of activin/inhibin showing absence of immunostaining in Leydig (arrow) and Sertoli cells (arrowhead). Original magnifications: $\times 200$ (A–F); $\times 500$ (G–I).

and infertility. In the other group, however, a less severe phenotype was observed. Although testis size of animals in this group was reduced by 50%, fertility was not impaired. The reason for variable response in suppression of spermatogenesis is obscure, but could be related to observations in men in which azoospermia was achieved only in 50 to 70% of men treated with various hormonal contraceptive regimens.⁵⁴

Smad4 is the co-Smad for activin, TGF- β , and BMP. Activins and inhibins, apart from regulating the pituitary FSH synthesis and secretion, play a role in spermatogonial proliferation and modulation of steroidogenesis, respectively.^{11,12} The absence of activin β A subunit leads to perinatal lethality; therefore, its role in spermatogenesis cannot be evaluated.⁵⁵ However, absence of activin receptor II caused a reduction in seminiferous tubule diameter, but no primary defects in germ cell population, suggesting that this receptor may affect the proliferation and differentiation of Sertoli cells.⁵⁶ In contrast, expres-

sion of BMP8 genes in male germ cells tightly correlates with the progression of spermatogenesis.⁵⁷ Male mice deficient in BMP8b showed increased apoptosis of germ cells eventually leaving Sertoli cells only. This led to testicular degeneration and infertility in these mice.⁵⁸ These effects in the testis of BMP8b null mutants are similar to the gross testicular changes (apoptosis, depletion of germ cells, degeneration of seminiferous tubules, Leydig cell hyperplasia) that we observed by overexpressing the *Smad4* gene. However, we do not know whether these effects of *Smad4* overexpression are because of interference with BMP, or activin-mediated signaling pathways.

Follistatin, an activin-bioneutralizing binding protein binds to the β -subunits of activin and blocks its action.⁵⁹ Follistatin may regulate the function of other TGF- β family members. Supporting this notion, follistatin-deficient mice⁵⁹ have skeletal defects reminiscent of BMP5-deficient mice, and high concentrations of follistatin can block the effects of BMP7 and BMP4 *in vitro*.^{60,61} It is also

Figure 6. Cauda epididymis of 6-week-old normal littermate and *Smad4* transgenic mice. **A:** Spermatozoa are detected in the lumen of normal littermate (arrow). **B:** *Smad4* transgenic mouse tubular diameter is smaller than the normal littermate and spermatozoa are absent. Original magnifications, $\times 200$.

known that BMP8b knockout male mice were infertile.⁵⁸ Guo and colleagues⁴⁷ suggested that overexpression of follistatin may block BMP8b signaling and that may cause infertility. These authors⁴⁷ also suggested that under physiological conditions follistatin might play a role in the maintenance of spermatogenesis by modulating the function of BMP8b in the mouse. In our study, neither activin subunits nor follistatin levels changed on a per cell basis. However, it could be possible that because of an increase in Leydig cell number, the total testicular follistatin levels rise and may contribute to the overall pathological changes in these animals.

Apart from disruption of spermatogenesis, the other effects observed in the testis of our *Smad4* overexpressed male mice were massive Leydig cell hyperplasia. It has been reported that deficiency of germ cells can cause changes in Sertoli cell gene expression in the degenerated tubules.⁵⁸ Leydig cells are responsible for the production of testosterone, which supports spermatogenesis through effects on Sertoli cells. In turn, Sertoli cell-derived proteins, such as the complex of TIMP-procathepsin L, regulate testosterone production by Leydig

Figure 7. Cellular apoptosis was identified by TUNEL analysis in cells of the seminiferous tubules of testis sections from 6-week-old normal littermate and *Smad4* transgenic mice. **A:** Normal littermate showing absence of apoptotic cells. **B:** Testis from *Smad4* transgenic mice showing numerous apoptotic germ cells (green staining). Original magnifications, $\times 200$.

cells.⁶² In the present study, Leydig cell hyperplasia, accompanied by a marked rise in testosterone levels, was observed in *Smad4* transgenic mice. These results indicate that *Smad4* overexpression not only affects spermatogenesis but also interferes with the androgen biosynthetic pathway. In contrast to increased testosterone levels, no significant changes in FSH levels were observed, suggesting that the phenotype is because of paracrine-acting factors rather than to disruption in endocrine hormone levels.

In summary, the overexpression of *Smad4* caused variable degrees of Leydig cell hyperplasia, apoptosis of germ cells, spermatogenic arrest, and a complete seminiferous tubular degeneration leading to infertility. Serum testosterone levels increased whereas FSH levels remain unchanged. These results suggest that overexpression of *Smad4* leads to testicular abnormalities and infertility supporting the hypothesis that TGF- β -like signaling pathways are carefully governed to ensure normal fertility.

Acknowledgments

We thank Brigitte Mann and the Northwestern University RIA Core for hormone measurements; Dr. Lynn Doglio

and the Northwestern University Transgenic Animal facility for generating *Smad4* transgenic mice; Dr. Doug Stocco, Texas Tech University, Lubbock, TX, for the STAR antibody; Jose Santiago for help with the preparation of a figure in this manuscript; and Jeff Wrana, Hospital for Sick Children, University of Toronto, Canada, for the *Smad4* expression construct.

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