

## Developmental and Stage-Specific Expression of Smad2 and Smad3 in Rat Testis

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**ABSTRACT:** Members of the transforming growth factor beta type (TGF $\beta$ ) superfamily and their receptors are expressed in the testis, and are believed to play important paracrine and autocrine roles during testicular development and spermatogenesis. The Smad proteins are downstream mediators for the family of TGF $\beta$  growth factors. Smad2 and Smad3 are associated with both TGF $\beta$  and activin signaling. However, very little is known about the expression and regulation of the Smad signaling proteins in the testis. In the present study, we have determined that Smad2 and Smad3 proteins are expressed in the postnatal testes of rats from 5 days to 60 days of age. Expression levels for both proteins are higher in young rats than in sexually mature rats. Smad2 and Smad3 messenger RNA levels parallel protein expression. Smad2 and Smad3 proteins are mainly

localized in the cytoplasm of meiotic germ cells, Sertoli cells, and Leydig cells. Smad3 protein is localized to the nucleus of preleptotene to zygotene primary spermatocytes in young rats. Both proteins are expressed throughout all stages of the cycle of seminiferous tubules but are expressed at their lowest levels at stages VII–VIII in the seminiferous epithelium of adult rats. The presence of these downstream mediators in these cell types supports a role for TGF $\beta$  and activin during spermatogenesis. The difference between the expression of Smad2 and Smad3 suggests that they may have different functions within the testis.

Key words: Spermatogenesis, development, growth factors.

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Spermatogenesis is a complicated and highly ordered process by which spermatogonia mature into spermatozoa. The regulation of spermatogenesis involves both endocrine and autocrine/paracrine mechanisms. Although endocrine regulation of this process is essential, it is becoming evident that many growth factors and cytokines are involved in local regulation of spermatogenesis. The transforming growth factor beta type (TGF $\beta$ ) superfamily is one group of these factors (Mauduit and Benahmed, 1996; Saez and Lejeune, 1996; Ethier and Findlay, 2001; Kretser et al, 2001).

The TGF $\beta$  superfamily includes TGF $\beta$ , activin, inhibin, Mullerian inhibitory substance, growth and differentiation factors, and the bone morphogenetic proteins. These factors play important roles in a wide range of biological effects such as cell growth, proliferation, morphogenesis, cell differentiation, and apoptosis (Attisano and Wrana, 1998). Many members of this family and their receptors are expressed in the testes. For example, Sertoli

cells produce both inhibin B, which suppresses the production of follicle-stimulating hormone (FSH) in the pituitary, and activin, which stimulates FSH production (Roberts et al, 1989; Moore et al, 1994; Walsh et al, 1998). Inhibin and activin may also be local regulators of testicular function. Activin receptors are present in Sertoli cells, Leydig cells, and germ cells in the testis (de Winter et al, 1992; Kaipia et al, 1992). Activin treatment stimulates Sertoli cell proliferation (Boitani et al, 1995), increases mitotic activity of spermatogonia (Mather et al, 1990), and inhibits human chorionic gonadotropin (hCG)-stimulated testosterone formation (Hsueh et al, 1987). TGF $\beta$  and TGF $\beta$  receptors are expressed in Sertoli cells, Leydig cells, and germ cells. Treatment with TGF $\beta$  inhibits gonadotropin actions on Sertoli cells and Leydig cells, and also inhibits proliferation of Leydig cells and germ cells (Saez, 1994; Olaso et al, 1997, 1998; Wrana, 1998). However, knowledge of downstream mediators of TGF $\beta$  function in the testis is limited.

TGF $\beta$  family members initiate their cellular actions via heteromeric transmembrane kinase receptor complexes. These complexes phosphorylate downstream signaling molecules known as Smads (Feng and Derynck, 1997). Smads are a group of proteins that transmit TGF $\beta$  signals from the cell surface receptor into the nucleus (Derynck et al, 1996). Currently, 8 different Smads have been identified in mammals. They can be subdivided into 3 distinct subclasses based on function: receptor-activated Smads

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(R-Smads; Smad1, Smad2, Smad3, Smad5, and Smad8), common mediator Smad (Co-Smad; Smad4), and inhibitory or antagonistic Smads (anti-Smads; Smad6 and Smad7) (Piek et al, 1999). Activated type I receptors associate with and phosphorylate specific R-Smads within the Mad homology domain 2. The phosphorylated R-Smad dissociates from the receptor and forms a heteromeric complex with the Co-Smad Smad4, and together the heteromeric complex translocates to the nucleus, where it mediates target gene responses. Anti-Smads function as antagonists by blocking R-Smads from interacting with activated type I receptors and hence preventing their phosphorylation (Wrana, 2000).

Downstream signaling proteins might be the determinants of different effects of TGF $\beta$  family members within the testis. However, very little is known about the expression and regulation of the Smad signaling proteins in the testis. The expression of Smad1 and Smad2 in mouse testicular germ cells has been reported (Zhao and Hogan, 1997; Wang and Zhao, 1999), but Smad3 expression has not yet been described in the testis. Smad2 and Smad3 are highly homologous receptor-activated Smads that have been associated with both TGF $\beta$  and activin signaling (Derynck et al, 1998). In the present studies we have determined the expression and cellular localization of these 2 important signaling molecules during testicular development as well as their stage-specific expression in the adult rat testis.

## Materials and Methods

### *Animals and Tissue Preparation*

All animal experiments were performed in accordance with National Institutes of Health (NIH) guidelines and with institutional approval. Sprague-Dawley rats were obtained from Hilltop Lab Animals (Pittsburgh, Pa) and housed in standard conditions. Testes were collected from rats at 5, 10, 15, 20, 25, 30, and 60 days of age. They were fresh-frozen for Western blot analysis and RNA analysis, or freshly embedded in OCT for fluorescent immunostaining, or fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS) and embedded in paraffin for immunohistochemistry. Seminiferous tubules were isolated from 60-day-old rats and maintained in enriched Krebs-Ringer bicarbonate buffer (Bellvé et al, 1977). The stage-dependent transillumination pattern was identified using a stereomicroscope and 2-mm segments were sequentially cut and flash-frozen beginning with the stage VIII/IX border identified by the distinct dark/light interface (Toppari and Parvinen, 1985; Kangasniemi et al, 1990). Each age point contained at least 6 animals and seminiferous tubule segments were isolated from at least 5 animals. All animals were asphyxiated with CO<sub>2</sub> and killed by cervical dislocation.

### *Western Blotting*

Testes and seminiferous tubule segments were homogenized in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 0.5 mM MnCl<sub>2</sub>,

0.5 mM MgCl<sub>2</sub>, 5 mM ethyleneglycotetraacetic acid, 0.2 mM phenylmethylsulfonyl fluoride, and 1% sodium dodecyl sulfate), then centrifuged for 20 minutes, and the supernatant was collected. Protein content was quantified with the bicinchoninic acid analysis kit (Pierce, Rockford, Ill). Fifteen micrograms of protein lysate from each sample was subjected to denaturing polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane (Amersham, Buckinghamshire, United Kingdom). The resultant membranes were blocked with Tris-buffered saline (TBS) containing 5% (w/w) fat-free dry milk at 4°C overnight, then washed with TBST (a mixture of TBS and 0.05% Tween-20) 3 times for 10 minutes, and then incubated for 2 hours at room temperature with 3  $\mu$ g/mL of specific rabbit polyclonal antibody against Smad2 or Smad3 (Zymed Laboratories, San Francisco, Calif). After washing with TBST 3 times for 10 minutes, the membranes were incubated with peroxidase-conjugated affinity-pure goat anti-rabbit immunoglobulin G (IgG; 1:125 000 diluted in 1% bovine serum albumin (BSA)/TBST; Jackson ImmunoResearch, West Grove, Pa) for 1 hour at room temperature. They were then washed with TBST 3 times for 10 minutes, and imaged with the ECLPlus Western blotting detection system (Amersham). Enhanced chemiluminescence-incubated blots were exposed to Hyperfilm (Amersham). Then membranes were washed with warm TBST and reprobed with actin antibody (mouse monoclonal, 1:1000 diluted in 1% BSA/TBST; Chemicon, Temecula, Calif) as an internal control.

### *Messenger RNA Analysis*

Total RNA from testes of animals at different ages was isolated with TRI-reagent (Molecular Research Center, Cincinnati, Ohio). Samples of total RNA (1–5  $\mu$ g) were analyzed for Smad2 and Smad3 message by RNase protection assay (RPA) using the RPA III kit according to the instructions provided by the supplier (Ambion, Austin, Tex). Antisense RNA probes were labeled with [ $\alpha$ -<sup>32</sup>P]CTP (800 ci/mmol) using the In Vitro Transcription kit (Ambion). The template complementary DNA constructs of rat Smad2 and Smad3 were kindly provided by Dr Mark Nachtigal (Dalhousie University, Halifax, NS, Canada). The specific activities of Smad2 and Smad3 probes were in the range of 2–8  $\times$  10<sup>7</sup> cpm/ $\mu$ g for the different experiments. Approximately 100 000 cpm was added per reaction for both Smad2 and Smad3 probes. The size of the protected Smad2 and Smad3 bands is 386 bp and 616 bp, respectively. Cyclophilin (111 bp) was used for internal control, and was kindly provided by Dr Anthony Zeleznik (University of Pittsburgh, Pittsburgh, Pa). Following electrophoresis, gels (5% acrylamide containing 8 M urea) were exposed to x-ray film for 24–72 hours.

### *Biotin-Avidin DCS System Indirect Immunofluorescence Techniques*

Frozen sections were cut, air-dried, and incubated with 10% goat serum/PBS (0.01 M) for 1 hour at 4°C in a humidified chamber. Specific rabbit polyclonal antibodies against Smad2 or Smad3 (diluted in 10% goat serum/PBS, 5  $\mu$ g/mL final concentration) were applied to the sections and slides were incubated overnight at 4°C. For negative control, the primary antibody was preabsorbed with 50  $\mu$ g/mL Smad2 or Smad3 peptide (Zymed) for 2 hours in working dilution. Slides were washed with cold PBS 3

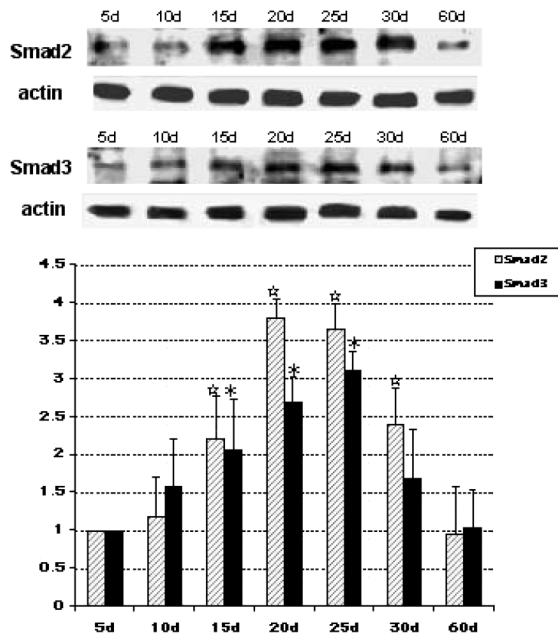


Figure 1. Western blot analysis of whole testis lysates (15  $\mu$ g protein) obtained from rats of different ages. Western blot analysis for Smad2 and Smad3 resulted in specific bands at 58 kd and 56 kd, respectively. Actin was used for the internal standard (43 kd). Bars represent relative expression of Smad2 and Smad3 proteins at each age point. Signals for Smad2 and Smad3 were standardized to the actin signal, then normalized to the 5th day of age, which was arbitrarily designated as 1. Each bar represents the average of relative band intensity from 6 experiments  $\pm$  SEM.  $\star$  Represents  $P < .05$  vs 5 days of age for Smad2; \*,  $P < .05$  vs 5 days of age for Smad3.

times for 10 minutes and biotinylated–anti-rabbit IgG antibody (1:200 diluted in 10% goat serum/PBS, Vector Laboratories, Burlingame, Calif) was applied to the tissue sections for 1 hour at 4°C. Slides were washed with cold PBS 3 times for 10 minutes and fluorescein avidin D cell sorter (1:200 in sodium bicarbonate buffer; Vector) was applied to the sections, and slides were incubated for 1 hour at 4°C. After washing with cold PBS, slides were incubated in 0.5  $\mu$ g/mL propidium iodide/PBS for 5 minutes, then washed with distilled water, and mounted with Vectashield mounting medium (Vector). The slides were digitally imaged (Optronics 3 CCD Digital/Analog Camera) using a Leica DMR fluorescence microscope using 510 nm and 580 nm filters, and images were overlaid using KS300/Axiovision software (Zeiss, Thornwood, NJ).

#### Immunohistochemistry

Six-micrometer-thick serial paraffin sections were cut. Sections were dewaxed in xylene and rehydrated, then boiled in a microwave oven in glycine buffer (3.75 g glycine and 0.1 g ethylenediamine tetraacetic acid (EDTA) in 1 L of distilled water pH 3.6) twice for 5 minutes, cooled at room temperature, and washed with distilled water. The sections were then immersed into 1% H<sub>2</sub>O<sub>2</sub>/methanol for 10 minutes, and washed with PBS. The sections were then incubated with 10% goat serum/PBS (0.01 M) for 1 hour at 4°C in a humidified chamber. Specific rabbit polyclonal antibodies against Smad2 or Smad3 (diluted in 5  $\mu$ g/mL of 10% goat serum/PBS; Zymed) were applied to the

sections, and slides were incubated overnight at 4°C. For negative control, the primary antibody was preabsorbed with 50  $\mu$ g/mL of Smad2 or Smad3 peptide (Zymed) for 2 hours in working dilution. Slides were washed with PBS and biotinylated–anti-rabbit IgG antibody (1:200 diluted in 10% goat serum/PBS; Vector) was applied to the tissue sections for 1 hour. Slides were washed in PBS and avidin-biotin peroxidase (ABC; Vector) was applied to the sections for 30 minutes at 4°C. After washing with PBS, the sections were incubated with diaminobenzidine solution (Zymed) for 5–10 minutes, then counterstained with Mayers hematoxylin for 30 seconds.

#### Data Analysis

All experiments were repeated at least 3 times. The intensities of protected RNA fragments and Western blotting signals were quantitated using a flatbed scanner image of the radiographic films followed by analysis with NIH Image software. Signals obtained from Smad2 and Smad3 hybridizations were corrected with actin signal, then normalized against signal from the testes of 5-day-old rats or stage I tubule segments. For RPA, densitometric signals from individual bands were normalized to cyclophilin to correct for differences in gel loading of the messenger RNA (mRNA) changes, or of its selectivity, or both.

Statistical significance between mean values was determined by analysis of variance followed by the Student-Neuman-Keuls test or the Dunnett test, and was accepted at the 0.05 level.

## Results

### Postnatal Developmental Expression Pattern of Smad2 and Smad3 in Rat Testis

We first investigated the postnatal developmental expression pattern of Smad2 and Smad3 proteins in the testes of rats of different ages. Western blot analysis for Smad2 and Smad3 resulted in specific bands at expected sizes for each Smad. As shown in Figure 1, both Smad2 and Smad3 proteins are expressed in the testis of rats from 5 days to 60 days of age. The expression level for Smad2 protein increases 2.2-fold to 3.8-fold in the testes of 15- to 30-day-old rats compared with the testes of 5-day-old rats ( $P < .05$ ). Smad3 protein increases from 1.7-fold to 3.1-fold at the same age points ( $P < .05$ ). Low expression levels for both proteins are observed at 60 days after birth.

To determine whether RNA levels correlated with expressed proteins for Smad2 and Smad3, we used RPA. As shown in Figure 2, Smad2 and Smad3 mRNA transcripts were detected at all age points examined. Smad2 transcript level increased by 1.5-fold to 2-fold in the testes of 10-day-old to 25-day-old rats compared with that of 5-day-old rats ( $P < .05$ ). The lowest transcript levels ( $P < .05$ ) were observed in testes of 60-day-old rats. The expression pattern for Smad3 had a similar trend as that for Smad2, but the differences did not achieve statistical significance, except at 60 days of age ( $P < .05$ ).

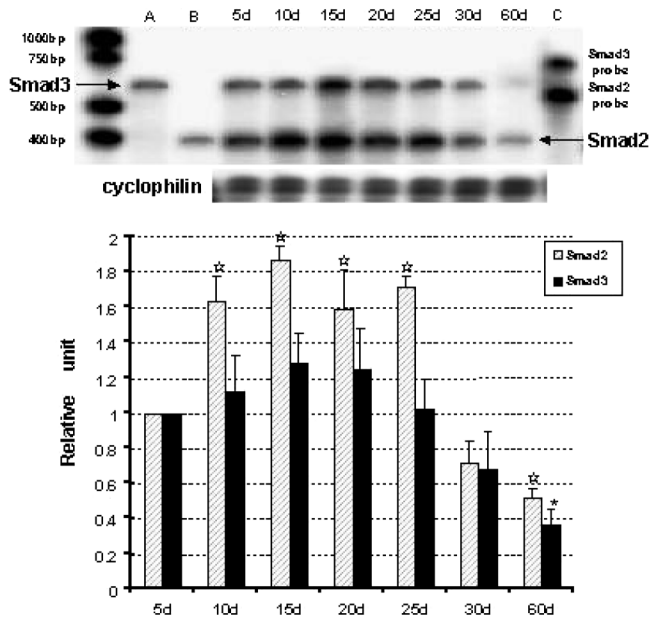


Figure 2. RPA for Smad2 and Smad3. Total RNA was isolated from testis of different age rats. Signals were corrected with cyclophilin and normalized to the 5th day of age, which was arbitrarily designated as 1. Bars represent the average of relative band intensity from 3 experiments  $\pm$  SEM. Lane A, Smad3 protected band. Testicular RNA hybridized with only Smad3 probe. Lane B, Smad2 protected band. Testicular RNA hybridized with only Smad2 probe. Lane C, Smad2 and Smad3 probes. ☆ Represents  $P < .05$  vs 5 days of age of Smad2; \*,  $P < .05$  vs 5 days of age of Smad3.

*Stage-Specific Expression of Smad2 and Smad3 in the Seminiferous Epithelium of Adult Rat Testes*

In the seminiferous epithelium, male germ cells in different developmental phases are arranged in defined associations or stages. Along the seminiferous tubules, these stages follow each other in a regular, consecutive, linear fashion, giving rise to the wave of the seminiferous epithelium in most mammals (Parvinen, 1982; Griswold, 1995). To determine the stage-specific expression patterns for the Smad2 and Smad3 proteins, seminiferous tubule segments from the testes of 60-day-old rats were isolated by the transillumination microdissection technique, and Western blot analysis was performed on segment extracts. Both proteins were expressed throughout all stages of the cycle of seminiferous tubules. However, the levels of Smad2 and Smad3 varied during the cycle of seminiferous epithelium. Specifically, expression of Smad2 and Smad3 declined in stages VII–VIII (Figure 3).

*Cellular Localization of Smad2 and Smad3 Proteins in Rat Testes*

To determine the cellular localization of Smad2 and Smad3 proteins in the testes, we performed fluorescent immunostaining on frozen sections from rats at postnatal days 5 to 60 as described in “Materials and Methods.” The positive staining for Smad2 and Smad3 appears

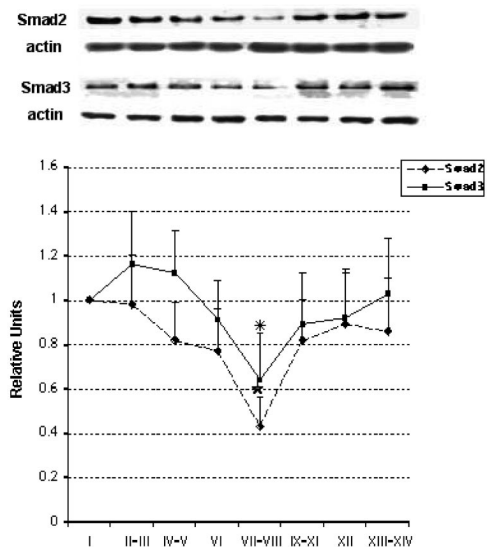


Figure 3. Western blot analysis of isolated segments of seminiferous tubule lysates (15  $\mu$ g protein). Signals were corrected with actin signal and normalized to stage I, which was arbitrarily designated as 1. The bars represent the average relative band intensity from 3 experiments  $\pm$  SEM. ☆ Represents  $P < .05$  vs stage I for Smad2; \*,  $P < .05$  vs stage I for Smad3.

green, and nuclei are stained red. Signals for both Smad2 and Smad3 proteins were observed in the seminiferous tubules at all age points we examined. As shown in Figure 4, in the testes of 10-day-old rats, both proteins were detected in the cytoplasm of both Sertoli cells and Leydig cells. No significant signal was observed in spermatogonia. In the testes of 25-day-old rats, Sertoli cells and Leydig cells continued to express both Smad2 and Smad3 in the cytoplasm. No obvious staining was observed in spermatogonia. Smad2 protein was detected in the cytoplasm of all meiotic germ cells, particularly pachytene spermatocytes. The Smad3 protein was also detected in the cytoplasm of all meiotic germ cells. In the testes of 60-day-old rats, minimal Leydig cell staining for either Smad2 or Smad3 protein was observed. Sertoli cells maintained their expression of Smad2 and Smad3. On the negative control sections in which the primary antibody was preabsorbed with Smad2 or Smad3 peptide, only the sperm and tubular lumen showed high background (Figure 4).

To clarify histological and morphological details, we performed ABC staining on the serial paraffin sections as described in “Materials and Methods.” For these studies, Smad immune complexes are indicated by brown staining. In the testes of 10-day-old rats, the staining was consistent with that shown in Figure 4. Smad2 protein was mainly located in the center of the tubules, which corresponds to the cytoplasm of Sertoli cells (Figure 5A); in contrast, Smad2 expression was not evident in spermatogonia (Figure 5C). Positive signals were detected in the

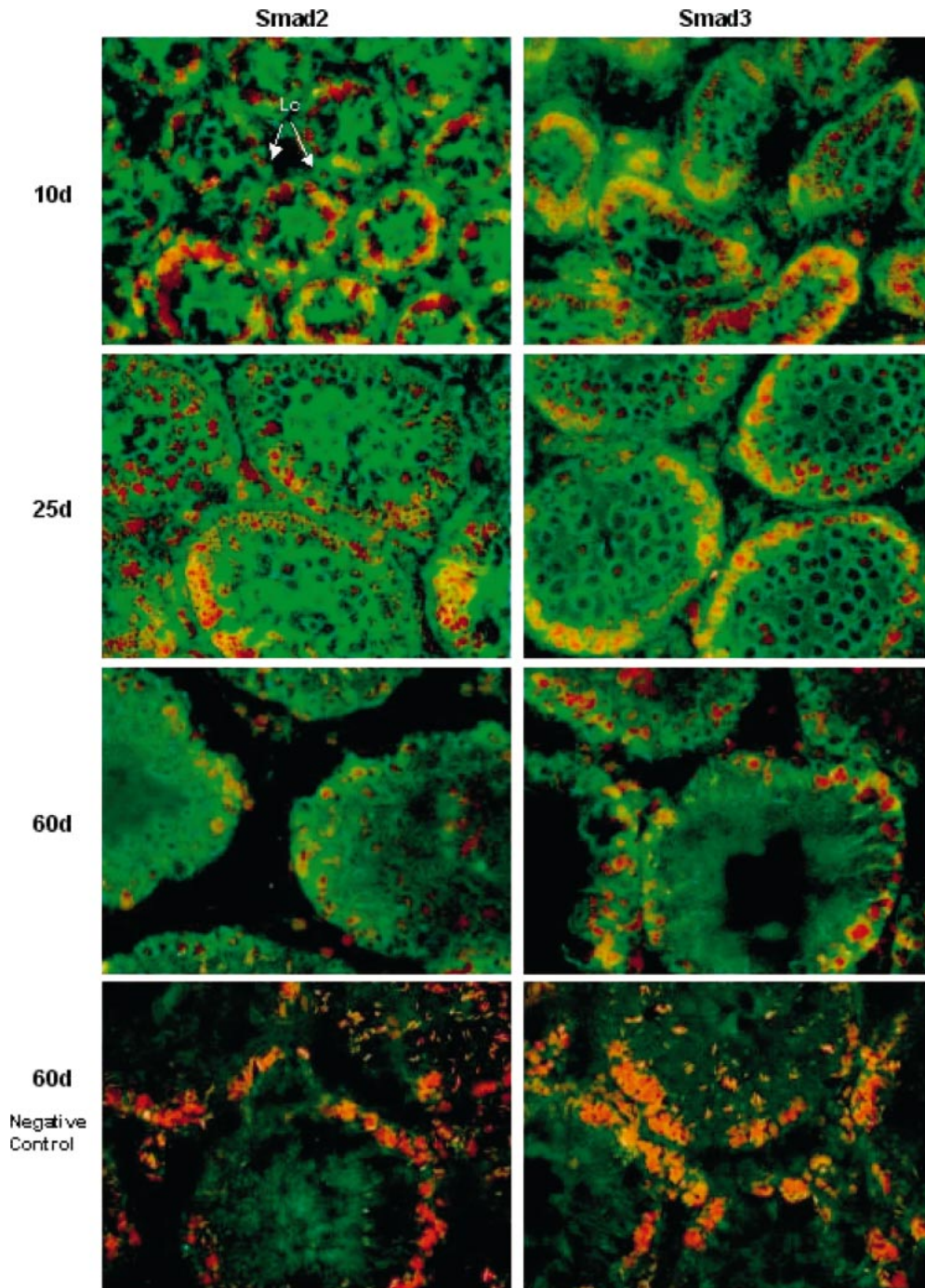


Figure 4. Fluorescent immunostaining of frozen sections of rat testis for Smad2 and Smad3. Propidium iodide was used to stain the nuclei. Slides were visualized at 200 $\times$  using a fluorescence microscope with 510 nm and 580 nm filters, and images were overlaid using KS300 software. The positive signals for Smad2 and Smad3 are green, and the nuclear staining is red. Negative control sections were stained with primary antibody preabsorbed with Smad2 or Smad3 peptide (bottom row). Lc indicates Leydig cells.

cytoplasm of Leydig cells outside the tubules (Figure 5A and C). Smad3 had a similar expression pattern in 10-day-old rats as shown in Figure 5B and D, a serial section from the same block as Figure 5A and C. At 25 days of age, Smad2 protein was expressed in the cytoplasm of meiotic germ cells, Sertoli cells, and Leydig cells (Figure

5E and G); no signal was observed in spermatogonia (Figure 5G). In adjacent sections stained for Smad3 (Figure 5F and H), some preleptotene, leptotene, and zygotene spermatocytes as well as Sertoli cells had nuclear staining, but no staining was evident in spermatogonia (Figure 5H). The negative control sections in testes of 10- and

25-day-old rats had negligible background staining (data not shown).

In the adult testis, immunohistochemistry revealed that both proteins are expressed in all stages of the cycle of seminiferous epithelium (Figure 5I and J), consistent with the Western blot analysis results (Figure 3). Sertoli cells at all stages continue to express both proteins in the cytoplasm. However, in stages VII–VIII, Sertoli cells exhibited nuclear staining for Smad3 (Figure 5L). Both Smad2 and Smad3 proteins were mainly detected in the cytoplasm of spermatocytes and were also detected in spermatids, whereas Leydig cells in adult testes had no obvious positive staining for either protein (Figure 5I and J). Neither Smad2 nor Smad3 was expressed in spermatogonia (Figure 5K and L). The adjacent negative control sections with primary antibody preabsorbed with Smad2 (Figure 5M) or Smad3 (Figure 5N) peptide produced negligible background staining.

## Discussion

Smad2 and Smad3 are highly homologous receptor-activated Smads that mediate signal transduction for both TGF $\beta$  and activin (Derynck et al, 1998). Smad2 has wide functions in regulating cell growth and differentiation (Kretzschmar and Massague, 1998; Padgett et al, 1998), and plays a very important role in early embryonic development (Nomura and Li, 1998; Waldrip et al, 1998; Weinstein et al, 1998). Smad3 was shown to have effects in wound healing (Zhu et al, 1998), and TGF $\beta$ -mediated regulation of T cell activation (Ashcroft et al, 1999), mucosal immunity (Yang et al, 1999), and ovarian folliculogenesis (Xu et al, 2002). However, expression and function of Smad2 and Smad3 are unknown during testis development.

In the present study we first investigated the postnatal testicular developmental expression pattern of Smad2 and Smad3 proteins and mRNA. Both proteins were expressed at all age points we examined. Expression levels for both proteins were higher in juvenile rats (ages 15–25 days postnatal) than in sexually mature rats (60 days of age). Smad2 and Smad3 mRNA levels paralleled protein expression. Smad2 and Smad3 proteins were localized in meiotic germ cells, Sertoli cells, and Leydig cells, but specific expression varied in juvenile and mature animals. Both proteins were expressed in a stage-specific manner in the seminiferous epithelium of adult rats.

In the rat testis, germ cells begin to develop about 2 weeks after birth. At 15–25 days after birth, meiotic germ cells are the most abundant cell type. By 30 days, elongating spermatids are present in the rat testis (Malkov et al, 1998). Higher expression of Smad2 and Smad3 from day 15 to day 30 suggests that these 2 proteins may be

involved in germ cell maturation. Both proteins are present in meiotic germ cells during this period (Figures 4 and 5), which supports this possibility. The level of activin type IIA receptor mRNA expression increases in rat testes from day 20 to day 30 (de Winter et al, 1992), and activin receptor mRNA has been localized to spermatogenic cells (Kaipia et al, 1992). Furthermore, a delay in puberty has been reported in male mice deficient in activin receptor (Matzuk et al, 1995). The presence of both Smad2 and Smad3 and activin receptor in the juvenile stage of development leads to consideration that this pathway may be involved in maturation of the first wave of germ cells. However, further study is necessary to determine whether Smads have a role in germ cell maturation. Between 15 and 30 days of age, Leydig and Sertoli cells also undergo proliferation and differentiation (Griswold, 1998; Mendis-Handagama and Ariyaratne, 2001). The high levels of expression of Smad2 and Smad3 during this time of development could be related to somatic cell regulation rather than to germ cell maturation.

In the current study, we found that both Smad2 and Smad3 were expressed in rat meiotic germ cells, Sertoli cells, and Leydig cells (Figures 4 and 5). However, Smad3 protein was localized to the nucleus of preleptotene to zygotene spermatocytes in young rats (Figure 5F and H). Translocation of R-Smads is a key event in the Smad signaling pathway. Following phosphorylation, Smads undergo translocation from the cytoplasm into the nucleus, where they mediate target gene transcription (Wrana et al, 2000). Nuclear localization suggests that Smad3 may be more actively involved in the regulation of early meiotic spermatocytes than Smad2. Further functional studies are necessary to definitively demonstrate the role of Smad3 in meiosis.

A previous report (Wang and Zhao, 1999) localized Smad2 protein by immunohistochemistry and mRNA by *in situ* hybridization to meiotic germ cells, Sertoli cells, and Leydig cells in the mouse. In contrast to our studies, that report did not find Smad2 in postmeiotic germ cells. We detected signal for both Smad2 and Smad3 in spermatids using both a fluorescent technique (Figure 4) and the peroxidase detection method (Figure 5). Like the previous study, we found a high level of Smad2 in meiotic germ cells, but we also demonstrated a low but specific signal for Smad2 in postmeiotic germ cells. It is possible that Smad2 expression is different in mice than it is in rats. However, we also used different antibodies and different blocking protocols and this may also account for the difference in detecting a low level of expression.

TGF $\beta$  and activin are produced by Sertoli cells, and their receptors are expressed in most testicular cell types. Activin treatment can stimulate Sertoli cell proliferation, and TGF $\beta$  treatment can inhibit gonadotropin action on Sertoli cells (Hsueh et al, 1987; Mather et al, 1990; de

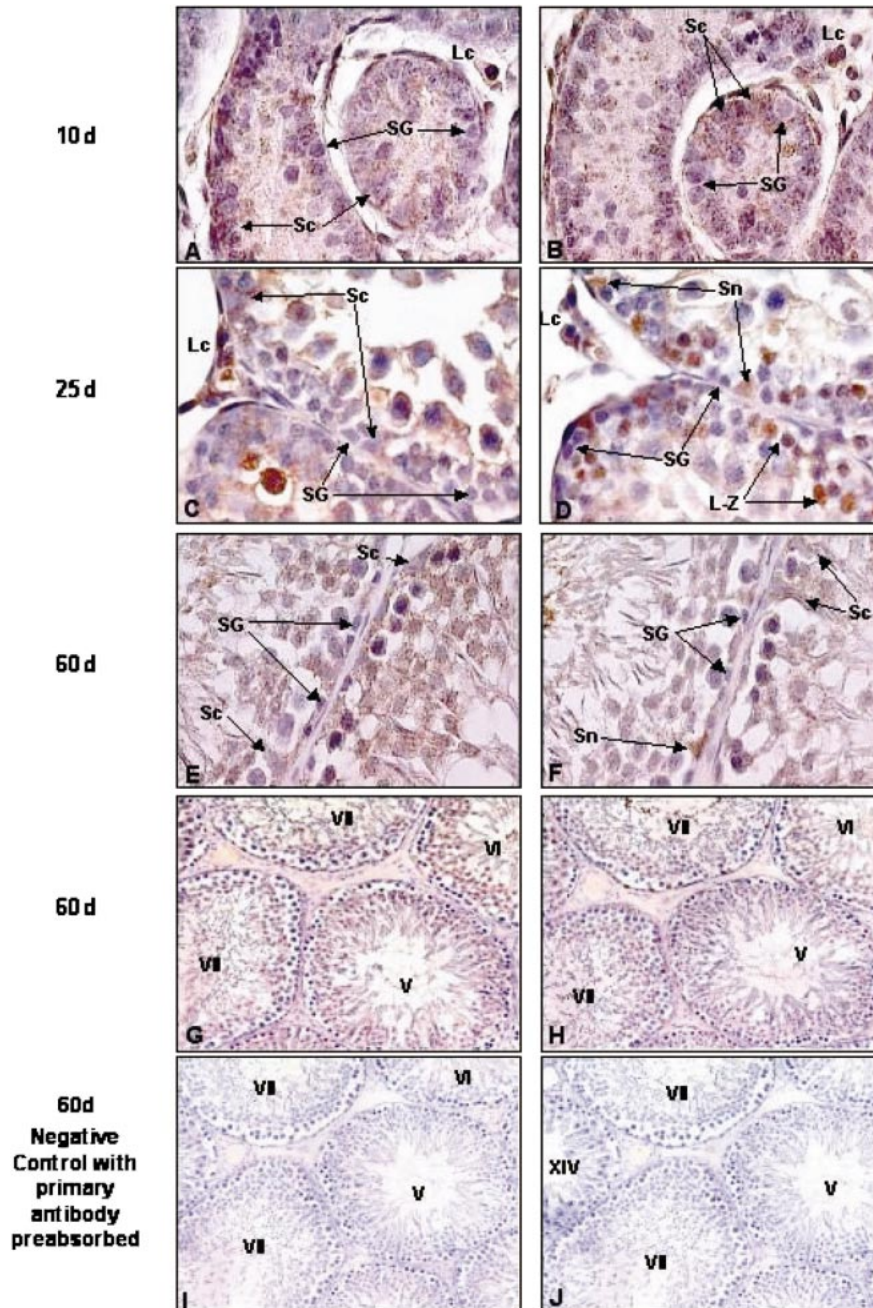


Figure 5. Immunohistochemical staining on the serial paraffin sections of rat testis for Smad2 (left column) and Smad3 (right column). The positive signal for Smad2 and Smad3 is brown. Ten days of age (A and B), 25 days of age (C and D), and 60 days of age (E–J). Panels (A–F) are 200 $\times$  magnification, (G–J) are 400 $\times$  magnification. Roman numerals represent the stage of seminiferous epithelium. Lc indicates Leydig cells; Sc, cytoplasm of Sertoli cell staining; Sn, nucleus of Sertoli cell staining; SG, spermatogonia; L-Z, leptotene-zygotene spermatocytes.

Winter et al, 1992; Kaipia et al, 1992; Boitani et al, 1995; Olaso et al, 1998), but the roles of these growth factors in Sertoli cells *in vivo* are still unclear. We have demonstrated that Sertoli cells continue to express both Smad2 and Smad3 throughout testicular development. The presence of these downstream signaling proteins of TGF $\beta$  and activin supports a role for TGF $\beta$  and activin

in Sertoli cells during testicular development and spermatogenesis.

Leydig cells express Smad2 and Smad3 in the prepubertal and pubertal testis, but their expression declines in the mature testis. As discussed above, this pattern correlates with the proliferation and differentiation of Leydig cells. Studies have reported that TGF $\beta$  inhibited the pro-

liferation and differentiation of Leydig cells and that activin inhibited hCG-stimulated testosterone production by Leydig cells in vitro (Saez, 1994; Olaso et al, 1997, 1998; Wrana, 1998). Cells in culture do not necessarily represent the behavior of cells in vivo. Granulosa cells of preovulatory follicles do not express Smad2 or Smad3 (Xu et al, 2002), yet cultured preovulatory granulosa cells can be induced to express both Smads. Further studies are needed to determine whether TGF $\beta$  and activin have specific roles in Leydig cell proliferation and differentiation in vivo.

Expression of Smad2 and Smad3 varies in the wave of seminiferous epithelium. Smad2 and Smad3 show their lowest expression levels at stages VII–VIII (Figure 3). Germ cells in stages VII–VIII of the wave undergo a multitude of profound metabolic changes and several alterations occur in the intercellular relationships. Considerable variation in gene expression occurs in these stages (Parvinen, 1982). Endogenous testosterone has maximal concentration in stages VII–VIII, when testosterone is required for spermatocyte survival and to allow progression through meiosis (Parvinen, 1982). The secretion rate of androgen-binding protein is also maximal in these same stages (Ritzen et al, 1982). Androgen receptor is also maximally expressed in the Sertoli cells in stages VII–VIII (Shan et al, 1997). Recently, Smad3 was found to function as a repressor of androgen receptor–mediated transcription (Hayes et al, 2001). The lowest level of Smad3 expression correlates with a point of maximal androgen action, suggesting that there may be interaction between androgen–mediated and growth factor–mediated regulation of germ cell maturation.

Sertoli cells function as nurse cells for germ cell maturation. The role of Sertoli cells changes as the germ cells progress through the stages of the cycle of the seminiferous epithelium (Parvinen, 1982). Sertoli cells show nuclear staining for Smad3 in stages VII–VIII (Figure 5L). During this time, primary spermatocytes move through the tight junction complexes between the adjacent Sertoli cells, whereas spermatids move centripetally, and are finally released from the epithelium at the end of stage VIII. The different pattern of intracellular localization of Smad2 and Smad3 during the wave of the seminiferous epithelium suggests that Smad2 and Smad3 proteins may be involved in different functions during spermatogenesis.

In conclusion, Smad2 and Smad3 are expressed in the Sertoli cells, Leydig cells, and meiotic germ cells over the course of testicular development and germ cell maturation. The presence of these downstream mediators in these cell types supports a role for TGF $\beta$  and activin during spermatogenesis. The difference between the expression of Smad2 and Smad3 suggests that they may have different functions within the testis.

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