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Effects of Short Photoperiod on the Expression of Smad2 and Smad3 mRNA in Syrian Hamster Testis

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ABSTRACT. The testicular localization and expression of Smad2 and Smad3 mRNA involved in the intracellular signal transduction of activin, inhibin and transforming growth factor-beta (TGF-β) were examined under the influence of long and short photoperiod in Syrian hamsters (*Mesocricetus auratus*). In situ hybridization detected both Smad2 and Smad3 mRNA in spermatogonia and premeiotic spermatocytes in the active testis exposed to a long photoperiod, as well as in the regressed testis exposed to a short photoperiod. Northern blots showed that Smad2 mRNA was expressed at all stages over long and short photoperiods, whereas Smad3 mRNA was expressed at high levels in the photoperiod-induced regressed testis. The photoperiodic condition would change the balance between Smad2 and Smad3 transcripts in the testis. Thus, intracellular Smad2 and Smad3 might participate in transducing signals from activin, inhibin and TGF-β in spermatogenetic cells.

KEY WORDS: hamster, photoperiod, Smad2, Smad3, testis.

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Activin, inhibin and transforming growth factor-β (TGF-β) have been implicated in the paracrine/autocrine regulation of mammalian spermatogenesis during the developmental and postnatal periods, because of their biological functions and localization [2, 7, 21, 31]. Activin promotes the number of germ cells in germ cell-Sertoli cell co-culture [21]. In contrast, inhibin reduces the numbers of rat and mouse spermatogonial cells after an intratesticular injection [31]. The synthesis of DNA is affected by TGF-β in organ cultures of the rat testis [7].

The Smad family is an important transducer of cytoplasmic signals from transmembrane receptors to the nucleus, upon stimulation by TGF-β [8, 19, 20]. Of the family, Smad2 [1, 5, 13, 18, 23] and Smad3 [3, 17, 23, 35] are activated by TGF-β and activin, while Smad1 [9, 13, 14, 16, 34] and Smad5 are activated by bone morphogenetic protein 2 (BMP-2) [34]. Phosphorylated Smads translocate to the nucleus and accumulate in response to TGF-β signaling [1, 9, 13, 18, 23], but the biological functions of the Smads in each tissue remain to be explored.

Gonadal activity in most seasonal breeders is mainly regulated by photoperiod. In response to an artificial alteration in photoperiod, testicular size and function change drastically in Syrian hamsters [6]. The TGF-β family is associated with testicular regulation in seasonal breeders. Testicular inhibin-α and βB subunit mRNA are significantly altered in the regressed testis exposed to a short photoperiod (SPP) and play important roles for the photoperiodic regulation of spermatogenesis [30]. However, the importance of Smads as intracellular mediators in the testicular regulation of the TGF-β family in seasonal breeders remains to be elucidated.

The present study examines the cellular localization of the Smad2 and Smad3 genes, which are involved in the intracellular signal transduction of activin/inhibin/TGF-β, in the testis of Syrian hamster. The relationship between photoperiod and expression of these genes in the testis of Syrian hamsters exposed to a short photoperiod is also investigated.

MATERIALS AND METHODS

Animals and conditions: Male Syrian hamsters (*Mesocricetus auratus*) maintained as a closed colony in the laboratory of Department of Veterinary Anatomy of the University of Tokyo were housed in a room with a long photoperiod (LPP) of 14L (cool white fluorescent tube light):10D until reaching sexual maturity at 8 weeks of age. They were bred in either a short photoperiod (SPP) of 6L:18D or a long photoperiod (LPP) of 14L:10D at a temperature of 23 ± 2°C. Food and water were provided *ad libitum*. Five to seven animals were statistically analyzed at 4, 7, 10 and 13 weeks after exposure to LPP or SPP. The hamsters were anesthetized with diethyl ether and sacrificed by cervical dislocation. The testes were rapidly removed, immersed in liquid nitrogen, weighed and stored at –80°C until RNA extraction. For *in situ* hybridization, the testes were perfused briefly with saline, followed by Bouin's fixative through the left ventricle and immersed in the same fixative overnight at room temperature. They were dehydrated in a graded series of ethanol, embedded in paraffin, cut at 5 μm and applied to silane-coated slides.

RNA isolation and northern blots: Total RNA was isolated from the testes by ultra-centrifugation on CsCl gradients [28]. The first strand of cDNA was prepared from 1 μg of total RNA using reverse transcriptase (Superscript II; Gibco BRL, MD) and an oligo dT primer. To prepare a specific cRNA probe for northern blotting and *in situ* hybridization, the polymerase chain reaction (PCR) was performed using the forward primer 5'-CAGCTTCTCTGAACAAACCAGG-3' and the reverse primer 5'-TACTCTGTGGCTCAATTCCTGCTG-3' for Smad2, the forward primer 5'-

CCAGCCATGTCGTCCATCCTGC-3' and the reverse primer 5'-CCCTTCCGATGGGACACCTGCA-3' for Smad3 and the forward primer 5'-TGAAGGTCGGTGTCAACGGATTTGGC-3' and the reverse primer 5'-CATGTAGGCCATGAGGTCCACCAC-3' for G3PDH. The fragments were inserted into the pGEM-T easy plasmid (Promega, WI) and sequenced. The plasmid used for *in vitro* transcription was digested with *Nco* I. A digoxigenin (DIG)-labeled ribonucleotide probe for Smad2 and Smad3 was synthesized by *in vitro* transcription in the presence of Sp6 RNA polymerase (Boehringer-Mannheim, Germany) at 37°C for 90 min. Total RNA (10 μg) was separated by electrophoresis on 1% agarose gels containing 2.2 M formaldehyde and transferred onto a Hybond N^+ membrane (Amersham, UK) by downward capillary blotting. The membrane was hybridized with the DIG-labeled RNA probe at 68°C for 16 hr, washed twice with 2×SSC containing 0.1% SDS at room temperature for 5 min, then washed with 0.1×SSC containing 0.1% SDS at 68°C for 1 hr. The membrane was incubated with an anti-DIG alkaline phosphatase-conjugated antibody and further processed to detect DIG chemiluminescence as recommended by the manufacturer (CDP-Star; Boehringer-Mannheim). The quantity of mRNA was verified by Northern blot using a G3PDH probe. Messenger RNA expression was quantified on X-ray film using NIH image software and the data are presented as pixels.

In situ hybridization: *In situ* hybridization was performed using a new method introduced by Boehringer-Mannheim. The testes were perfused with Bouin's fixative and sectioned at 5 μm. Deparaffinized sections were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min and incubated twice in 0.1% diethylpyrocarbonate (Sigma, MO) for 15 min. The sections were prehybridized in 50% formamide with 5×SSC containing 40 μg/m*l* sonicated salmon sperm DNA at 58°C for 2 hr and hybridized at 58°C for 40 hr with DIG-labeled antisense or sense riboprobes for Smad2 and Smad3. After hybridization, the sections were washed in 2×SSC at room temperature for 30 min and at 58°C for 30 min, in 0.1×SSC at 58°C for 30 min, then incubated at room temperature for 2 hr with alkaline phosphatase-coupled anti-digoxigenin antibody (Boehringer-Mannheim) diluted 1:2000 in buffer 1 (Tris-HCl 100 mM, NaCl 150 mM, pH 7.5) containing 0.5% blocking reagent (Boehringer-Mannheim). After washing in buffer 1, sections were developed at room temperature in buffer 2 (Tris-HCl 100 mM, NaCl 100 mM and $MgCl_2$ 50 mM, pH 9.5) containing nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Boehringer-Mannheim).

Statistics: Results were statistically analyzed by means of ANOVA followed by a *t*-test for evaluating statistical differences between experimental groups. Differences were considered significant at $P<0.05$.

RESULTS

Morphological and weight changes of testis: Spermatogenesis was active in the seminiferous tubules of sexually mature Syrian hamsters (8 weeks old). At 7 weeks after exposure to SPP, the lumen of some seminiferous tubules was closed, and spermatogenesis was interrupted. At 13 weeks, spermatogonia and spermatocytes were predominant in the seminiferous epithelium, but no spermatozoa were recognized. The diameter of the seminiferous tubules was significantly diminished and spermatogenesis was arrested. The ratio of testicular weight to the body weight of adult Syrian hamsters decreased with exposure to SPP and reached a minimum at 7 weeks after exposure (Fig. 1). In contrast, the ratio of testicular weight did not significantly change in hamsters exposed to LPP (Fig. 1). After 13 weeks, the hamsters exposed to SPP had become insensitive to photoperiod, and their testicular weights gradually increased (data not shown).

Testicular localization of Smad2 and Smad3 mRNA: To determine the cellular localization of Smad2 and Smad3 mRNA in the testes of Syrian hamsters, we hybridized sections of testes with DIG-labeled antisense and sense riboprobes for Smad2 and Smad3 mRNA. At 8 weeks post partum, DIG-labeled antisense Smad2 and Smad3 cRNA probes were detected in the spermatogonia and premeiotic spermatocytes of control testes (Fig. 2A and C). In the testes of the hamsters exposed to SPP, the DIG-labeled antisense Smad2 cRNA probe was detected in spermatogonia and preleptotene spermatocytes at 13 weeks after exposure (Fig. 2E and I). At this point, the cellular localization of Smad3 mRNA antisense was similar to that of Smad2 mRNA (Fig. 2G). No signals were detected in the sense control (Fig. 2B, D, F and H).

Expression of Smad2 and Smad3 mRNA: We examined Smad2 and Smad3 mRNA expression in the testis exposed to

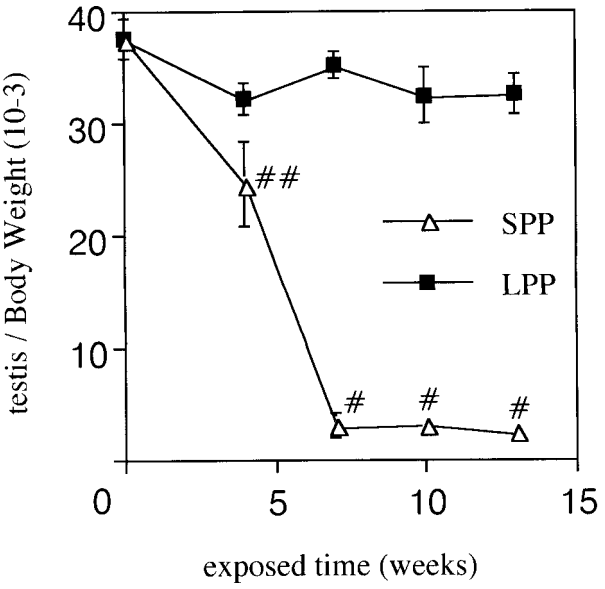

Fig. 1. Ratio of testis weight to body weight in the Syrian hamsters exposed to either long photoperiod (■; LPP, 14L: 10D) or short photoperiod (△; SPP, 6L: 18D). Data are expressed as means ± S.E. (n=5–7 animals per group), # $P<0.01$, ## $P<0.05$ vs. same exposure period.

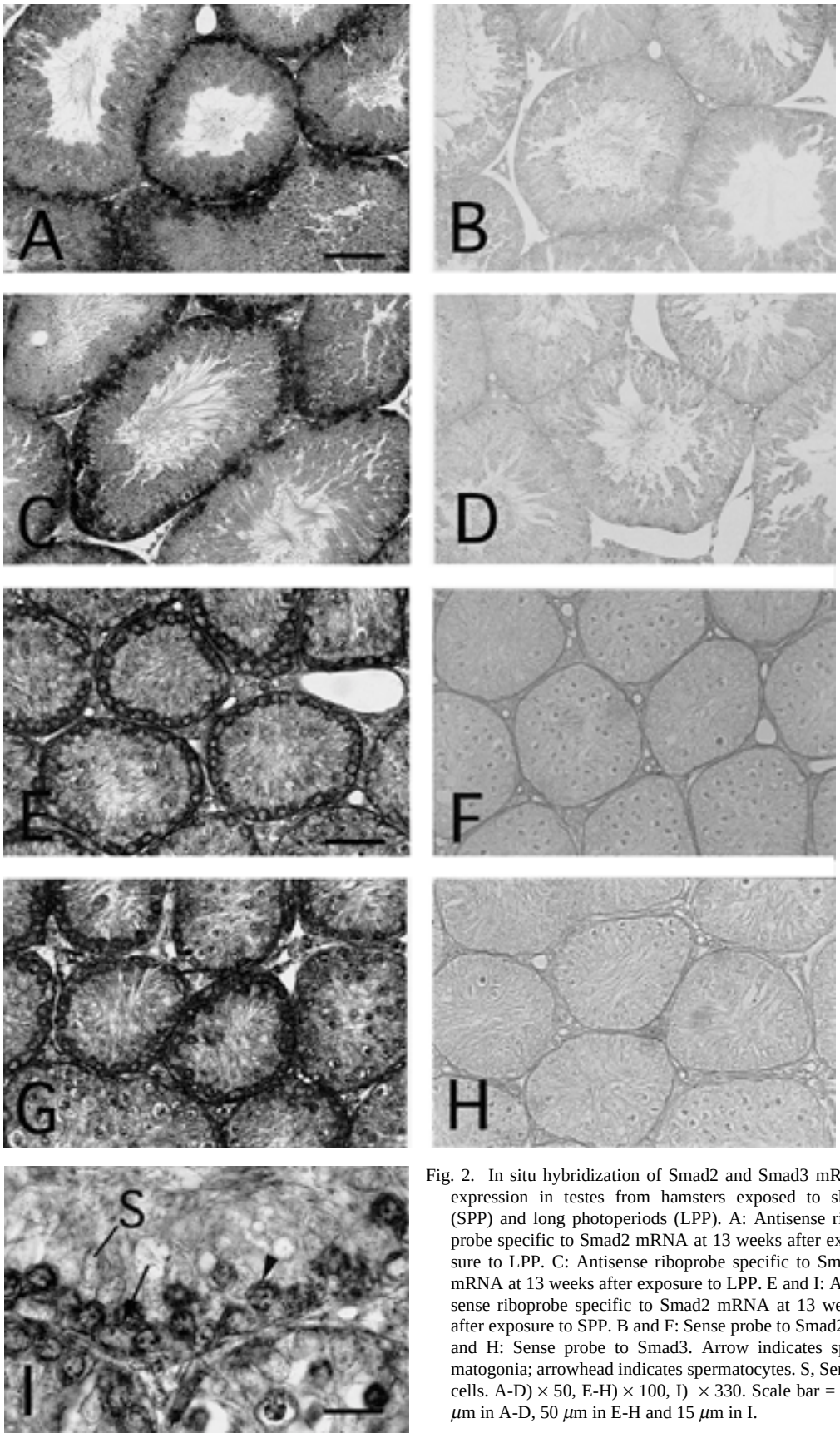

Fig. 2. In situ hybridization of Smad2 and Smad3 mRNA expression in testes from hamsters exposed to short (SPP) and long photoperiods (LPP). A: Antisense riboprobe specific to Smad2 mRNA at 13 weeks after exposure to LPP. C: Antisense riboprobe specific to Smad3 mRNA at 13 weeks after exposure to LPP. E and I: Antisense riboprobe specific to Smad2 mRNA at 13 weeks after exposure to SPP. B and F: Sense probe to Smad2. D and H: Sense probe to Smad3. Arrow indicates spermatogonia; arrowhead indicates spermatocytes. S, Sertoli cells. A-D) × 50, E-H) × 100, I) × 330. Scale bar = 100 μm in A-D, 50 μm in E-H and 15 μm in I.

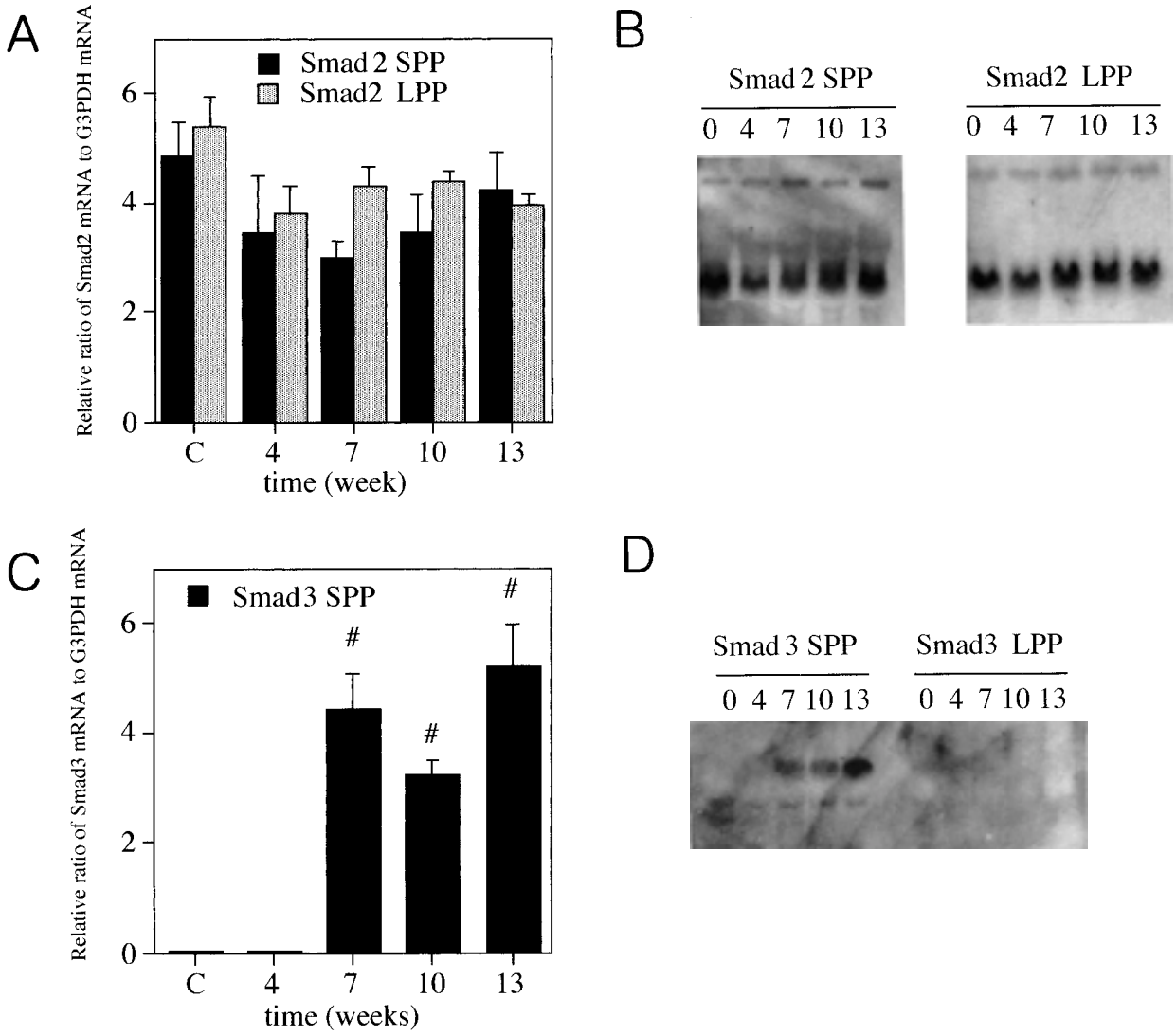

Fig. 3. Relative ratio of Smad2 and Smad3 mRNA to G3PDH mRNA in Syrian hamster testis exposed to long photoperiod (LPP; 14L:10D) and short photoperiod (SPP; 6L:18D) determined by Northern blotting. A and C: Relative ratio of Smad2 and Smad3 mRNA to G3PDH mRNA. B and D: Representative results of Northern blots using DIG-labeled cRNA probe specific for Smad2 and Smad3 mRNA (one animal per lane). Total RNA (10 μg) was Northern blotted. Data are expressed as means ± S.E of 5–7 observations. $^{\#}P<0.01$ vs. same exposure period.

SPP and LPP, by Northern blotting. Smad2 mRNA was expressed at the same level over the whole period during both SPP and LPP (Fig. 3A and B). On the other hand, the expression level of Smad3 mRNA was very low during LPP, but high during SPP at 7 weeks after exposure and this persisted until 13 weeks (Fig. 3C and D). Syrian hamster Smad2 mRNA, with two major transcripts of about 10 kb and 3 kb, was expressed at the same level over the whole period during both SPP and LPP with no significant differences (Fig. 3B). A major Smad3 transcript of about 6 kb was observed (Fig. 3D). Similar results were obtained in several independent experiments.

DISCUSSION

The Smad family mediates the TGF-β family in cytoplasm [8, 19, 20]. Among them, Smad2 and Smad3 are involved in signal transduction following activation of the receptors of activin, inhibin and TGF-β [1, 3, 5, 13, 17, 18, 23, 35], but their functions in spermatogenesis remain to be elucidated. In the adult mouse, Smad2 mRNA has been detected in premeiotic spermatocytes [32]. The present study localized both Smad2 and Smad3 mRNA in spermatogonia, and spermatocytes before meiosis in both active and regressed hamster testes. The cellular localization of Smad2 and Smad3 mRNA is in accordance with localization of TGF-β receptors in sper-

matogonia and spermatocytes [24] and of activin receptor mRNA (ActRIIB) in spermatogonia [10]. Thus, Smad2 and Smad3 might be involved in the signal transduction of premeiotic germ cells.

Northern blotting showed that these hamster Smad mRNAs were expressed in the testis, with many transcripts, the function of which *in vivo* has been unclear. The mRNA for Smad2 was expressed stably at all stages over both photoperiods, but Smad3 mRNA was expressed at high levels in the photoperiod-induced regressed testis. If such limited expression in spermatogonia and spermatocytes is considered, the Northern blot results may be interpreted differently. The population of spermatogonia and spermatocytes in the seminiferous tubules is elevated in the short photoperiod induced regressed testes. The expression of Smad3 mRNA still predominantly increased, but that of Smad2 mRNA might be reduced. Smad2 and Smad3 may have similar biological functions because the deduced sequences of their amino acids are highly homologous. Our Northern blot results revealed that the balance between Smad2 and Smad3 transcripts was altered by photoperiod. These results indicated Smad2 and Smad3 have different functions in the seasonal cycle of physiological states despite their structural similarity. Several investigators have found that levels of hormonally secreted inhibin are significantly reduced in the regressed testis of seasonal breeders [12, 15, 22, 27, 29]. Testicular regression induced by photoperiod significantly affects the expression of testicular inhibin α and βB mRNA in the bank vole [30] and in Siberian hamsters [25]. Activin, inhibin and Smads as intracellular mediators may have autocrine/paracrine roles in the regressed testis of these seasonal breeders.

Northern blots showed that the testicular expression profile of PAI-1 (Plasminogen Activator Inhibitor-1) mRNA, an index of the transduction of TGF-β and Smads [4, 11, 26, 33], concurred with that of Smad3 mRNA in testes exposed to SPP (data not shown). Thus, these Smads might mediate signals from TGF-β in germ cells to the nucleus. On the other hand, seem Smads probably do not mediate direct signals from activin in the regressed testis, because activin increases the number of spermatogonia and promotes DNA synthesis [7, 21, 31]. Considering the elevated expression of inhibin in the regressed testis [25, 30], further studies may be necessary to distinguish how Smads transmit signals from activin, inhibin and TGF-β.

In conclusion, this study found that Smad2 and Smad3 mRNA are localized in the spermatogonia and premeiotic spermatocytes of Syrian hamster testes exposed to SPP and LPP. Northern blotting revealed that Smad2 mRNA expression was stable at all stages over LPP and SPP, whereas Smad3 mRNA was increasingly modified in the regressed testis exposed to SPP. How these Smad mRNAs are regulated by photoperiod is currently unknown, but they may participate in signal transduction from activin, inhibin and TGF-β in Syrian hamster testicular cells.

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