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Expression of *Smad2* and *Smad4*, transforming growth factor- β signal transducers in rat endometrium during the estrous cycle, pre-, and peri-implantation

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Abstract

SMADs are intracellular signaling molecules that transmit signals elicited by members of transforming growth factor- β (TGF- β) superfamily. To decipher the mechanism of TGF- β signaling during the estrous cycle and implantation, we performed in situ hybridization to investigate the expression patterns of mRNAs for *Smad2* and *Smad4* in rat endometrium during the estrous cycle and on Days 0.5, 1.5, 2.5, 3.5, 4.5, 5.5, and 6.5 of pregnancy. Intense epithelial expression of *Smad2* mRNA at diestrus and proestrus was reduced at estrus and metaestrus, while *Smad4* maintained its constitutive expression during the estrous cycle. During pre-implantation, both *Smads* were accumulated in the luminal epithelium and the glandular epithelium. Contrary to the dramatic *Smad4* expression, *Smad2* was highly down-regulated on Day 2.5 and was increased on Day 3.5. During peri-implantation, both *Smads* were expressed in the luminal epithelium, subepithelial stroma, and the primary decidual zone. *Smad4* was down-modulated on Day 5.5. These results suggest that (a) both *Smads* are involved in the tissue remodeling of cycling and pregnant rat uteri; (b) TGF- β signaling functions mainly in the epithelium during pre-implantation and *Smad2* is involved in the endometrial switch from the neutral phase to the receptive phase; (c) TGF- β signaling is down-regulated at the time when trophoblast invasion begins and both *Smads* are involved in the formation of the primary decidual zone.

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1. Introduction

The mammalian uterus is a highly dynamic organ that undergoes extensive remodeling during normal estrous/menstrual cycle and pregnancy under hormone control. The degradation and re-synthesis of the extracellular matrix (ECM) components, which are essential for the endometrial breakdown and remodeling, could be affected by a variety of proteinases, cytokines, and growth factors (Tabibzadeh, 2002). Transforming growth factor- β (TGF- β) and related factors are multifunctional proteins that regulate cell proliferation and differentiation, ECM modification, tissue remodeling, angiogenesis, and immunosuppression. All of these events occur during the estrous cycle and embryo implantation. The TGF- β superfamily comprises TGF- β s, activins/inhibins, bone morphogenic proteins (BMPs), growth differentiation factors (GDFs), Müllerian inhibitory substance, etc. The ligands of the TGF- β superfamily transmit their signals through binding to type II receptor and then successively phosphorylating type I receptor, and both receptors are transmembrane serine/threonine kinases. SMAD proteins, recently discovered as downstream elements of the receptors, are intracellular signaling molecules that transmit the TGF- β signals into the nucleus, where they participate in the activation of downstream genes (Massagué, 1998).

SMADs have thus far been classified into three subclasses in vertebrates: receptor-regulated SMADs (R-SMADs, e.g. SMAD1, SMAD2, SMAD3, SMAD5, and SMAD8), common-partner SMADs (Co-SMADs, e.g. SMAD4 and SMAD4 β), and inhibitory SMADs (I-SMADs, e.g. SMAD6 and SMAD7). Upon receptors phosphorylation, R-SMADs form oligomeric complexes with Co-SMADs and then translocate into the nucleus where they regulate the transcription of target genes. SMAD2 and SMAD3 respond to TGF- β s and activins whereas SMAD1, SMAD5, and SMAD8 transmit BMPs signals. I-SMADs have been identified as antagonists of TGF- β signaling through stably interacting with phosphorylated type II receptors and preventing R-SMADs from being activated by these receptors (Massagué, 1998; Wrana and Attisano, 2000).

Numerous studies have revealed that in mouse (Tamada et al., 1990; Manova et al., 1992; Das et al., 1992), rat (Chen et al., 1993), mare (Lennard et al., 1995), bovine (Munson et al., 1996), swine (Gupta et al., 1996, 1998a, b), and human (Graham et al., 1992) endometrium or placenta, TGF- β s exhibit spatiotemporally different expression patterns, consistent with a role in implantation. Besides, other members of TGF- β superfamily, BMPs (Ying and Zhao, 2000; Paria et al., 2001) and activins (Manova et al., 1992; Albano et al., 1994; Otani et al., 1998; Jones et al., 2000), have also been proved to function in uterine tissue remodeling during decidualization. Strong evidence has accumulated that activin-A plays autocrine and paracrine roles in regulating trophoblast cell differentiation and placental hormone production (Peng and Mukai, 2000). Moreover, data from many laboratories have suggested that TGF- β receptors act in concert with TGF- β ligands in pregnant mouse decidua (Roelen et al., 1994), porcine conceptus (Gupta et al., 1996) and uterus (Gupta et al., 1998b), and human blastocyst (Osterlund and Fried, 2000), so that functional bioactive TGF- β s for embryo implantation are in evidence. The evidence (Fata et al., 2000) to date has indicated that TGF- β s may play maternal and placental inhibitory function on implantation. Although their exact function is still unclear, current evidence has suggested that TGF- β s may restrain trophoblast invasion through inhibiting the secretion of matrix metalloproteinases

(MMPs) (Bruner et al., 1999) and inducing the expression of tissue inhibitors of MMPs (TIMPs) (Graham and Lala, 1991, 1992; Huang et al., 1998), which are thought to be key proteases responsible for the degradation of ECM and inhibitory regulators of MMPs, respectively.

Regarding that the actions of TGF- β ligands are determined by their transmembrane receptors and intracellular signal transducer SMADs, details of the distribution patterns of these signaling pathway members could support the concept that TGF- β signal transduction pathways are operational. Unlike our knowledge regarding how TGF- β s and their receptors function in embryo implantation, far less has been reported what roles SMADs may play in this tightly regulated process, despite their important roles. Investigations of targeted gene disruption have put *Smads* in pivotal places in multiple biological processes in mammalian development (Weinstein et al., 2000). Thus far, a picture is also emerging whereby *Smad* molecules act in a cell type-specific manner in several reproductive events, such as spermatogenesis (Wang and Zhao, 1999), the folliculogenesis (Drummond et al., 2002; Findlay et al., 2002; Pangas et al., 2002), and the maturation of the oocyte and development of pre-implantation embryo (Osterlund and Fried, 2000). *Smad1* mRNA expression in mouse decidua has so far been the only documented case of *Smad* family expression in mammalian peri-implantation uterus (Ying and Zhao, 2000).

To gain further insight into the mechanism of TGF- β signaling in the estrous cycle and embryo implantation, we have therefore, examined rat cycling, pre-, and peri-implantation endometrium for the localization of *Smad2* and *Smad4*, which represent the intracellular signaling molecule of TGF- β s/activins and the shared partner of pathway-restricted *Smads*, respectively, using in situ hybridization.

2. Materials and methods

2.1. Animals and tissue preparation

All procedures involving animals were carried out in accordance with the Guidelines on the Care and Use of Laboratory Animals of the Ethical Committee, State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences. Adult male and female Sprague-Dawley rats weighing 200–250 g were used for the experiment. They were bred under a temperature- and light-controlled environment (temperature maintained at 25 °C, lights on from 07:00 to 19:00 h) with free access to food and water in Experimental Animal Center of Institute of Zoology, Chinese Academy of Sciences. The estrous cycle was staged by examining vaginal smears (Gouon-Evans and Pollard, 2001). To set up mating, a fertile male rat was caged with three virginal rats overnight, and the vaginal plug was checked between 08:00 and 09:00 h the next morning. Days 0 and 1 of pregnancy correspond to the midnight on the day the rats were mated and the following midnight on the day the vaginal plug was observed. Pregnant female rats were sacrificed at noon on Days 0.5, 1.5, 2.5, 3.5, 4.5, 5.5, and 6.5 of pregnancy ($n = 3$ for each time point), respectively. Uteri from cycling and pregnant rats were immediately removed, cleaned of fat tissues, cut into pieces, and embedded in embedding medium

(Triangle Biomedical Sciences, Durham, NC) at -20°C . Ten micrometer thick sections were cut transversely and mounted onto slides pre-coated with poly-L-lysine for in situ hybridization.

2.2. Hybridization probes synthesis and concentration determination

The cDNAs encoding SMAD2 and SMAD4 with C-Flag-tagged cytomegalovirus promoter-driven mammalian expression vector pRK5 (Zhang et al., 1996) were generous gifts from Dr. Zhijie Chang (Institute of Genomics, Tsinghua University, Beijing, China) and Dr. Rik Derynck (Department of Growth and Development, University of California, San Francisco, CA, USA). The two plasmids were digested with *EcoRI* and *HindIII*, which cleave the full-length *Smad2* and *Smad4* cDNAs. After purifying the cDNAs from the agarose gel using NucleoTrap[®] Gel Extraction Kit (CLONTECH Laboratories Inc., Palo Alto, CA, USA), the two cDNAs were inserted into the pGEM-3Z vector (Promega, Madison, WI, USA). Further, *EcoRI* and *HindIII* restriction digestion was conducted to confirm successful plasmids subcloning. Detailed information on the plasmid construction will be provided upon request. Sense and antisense single-stranded RNA probes were synthesized from linearized plasmids using digoxigenin (DIG) RNA labeling kit (SP6/T7) (Boehringer-Mannheim, Indianapolis, IN, USA). After ethanol precipitation and resuspension in DEPC-treated water, the yield of the probes was determined using spot test following the instructions of the manufacturer with a DIG-labeled RNA control which is provided in the labeling kit. Spot intensities of the control and experimental RNA probes were quantified using Band Leader version 3.0 (Magnostec Ltd., Tel Aviv, Israel) to estimate the concentration of the experimental probes.

2.3. In situ hybridization

In situ hybridization was carried out as described previously (Wang et al., 2001). Briefly, after being fixed in 4% paraformaldehyde for 15 min, the cryosections were incubated in PBS containing 0.1% active diethylpyrocarbonate (DEPC) twice for 15 min each, and in $5 \times$ sodium saline citrate (SSC; $1 \times$ SSC is 0.15 M NaCl, 0.015 M sodium citrate) for 15 min. The slides were then prehybridized in prehybridization solution (50% deionized formamide, $5 \times$ SSC, 120 $\mu\text{g/ml}$ salmon sperm DNA) for 2 h at 50°C , and hybridized in prehybridization solution containing denatured probes at a dilution of 400 ng/ml for 18 h at 50°C . The slides were then serially washed in $2 \times$ SSC at room temperature for 30 min, in $2 \times$ SSC at 65°C for 1 h, and in $0.1 \times$ SSC at 65°C for 1 h. Incubation with anti-DIG-alkaline phosphatase (diluted 1:5000 in blocking solution (0.5% blocking reagent in 100 mM Tris, 150 mM NaCl, pH 7.5); Roche Diagnostics Ltd., Hong Kong, China) at room temperature for 2 h was then followed. The slides were rinsed in washing buffer (100 mM Tris, 150 mM NaCl, pH 7.5) twice for 15 min each. Color development was performed using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT-BCIP; Boehringer-Mannheim). The sense probes were used as negative controls for background levels.

3. Result

3.1. Spatiotemporal distribution patterns of *Smad2* and *Smad4* mRNAs in rat endometrium during the estrous cycle

Results demonstrated distinct hybridization signals for *Smad2* and *Smad4* mRNAs in the luminal epithelium and the glandular epithelium of rat normal cycling endometria. Signals in the blood vessel, myometrium (data not shown), and the stroma were barely detectable. Intense *Smad2* mRNA accumulation was noted in the luminal epithelium and glandular epithelium at diestrus (Fig. 1a). This high expression level was sustained at proestrus (Fig. 1b), but was reduced at estrus and metaestrus (Fig. 1c and d). Unlike *Smad2*, *Smad4* mRNA was constitutively expressed in the cycling uteri. The intensity of staining in the luminal epithelium and the glandular epithelium during diestrus (Fig. 1e), proestrus (Fig. 1f), estrus (Fig. 1g), and metaestrus (Fig. 1h) was indistinguishable. The results are summarized in Table 1. No specific staining was detected when cycling uterine sections were hybridized with a sense probe (data not shown).

3.2. Spatiotemporal distribution patterns of *Smad2* and *Smad4* mRNAs in rat endometrium during pre-implantation

The luminal epithelium and the glandular epithelium are the major sites of localization of mRNAs encoding *Smad2* and *Smad4* in the pregnant rat uteri during pre-implantation period (Table 2). The luminal epithelium and the glandular epithelium exhibited intense accumulation of *Smad2* mRNA on Day 0.5 of pregnancy (Fig. 2a). This high hybridization levels in the luminal epithelium and the glandular epithelium were slightly reduced on Day 1.5, but the stromal cells adjacent to the myometrium in two samples showed clear staining (Fig. 2b). Reactions became weak in the luminal epithelium and the glandular epithelium on Day 2.5 (Fig. 2c). On Day 3.5, a distinct accumulation of *Smad2* mRNA was again noted in the luminal epithelium and the glandular epithelium (Fig. 2d). Staining in the stromal cells on Days 0.5, 2.5, and 3.5 was hardly detectable (Fig. 2a, c, and d).

The overall expression levels of *Smad4* transcripts in the pre-implantation rat uteri were large, compared with *Smad2*. The noteworthy strong signals in the luminal epithelium and the glandular epithelium remained at steady-state level on Days 0.5, 1.5, 2.5, and 3.5 (Fig. 2e and h), except for a decrease in the luminal epithelium on Day 1.5 (Fig. 2f).

Signals were hardly detectable in the blood vessel and myometrium (data not shown). Sense hybridization for both *Smad2* and *Smad4* showed negative results (data not shown).

3.3. Spatiotemporal distribution patterns of *Smad2* and *Smad4* mRNAs in rat endometrium during peri-implantation

Moderate levels of *Smad2* hybridization signals were observed in the luminal epithelial cells, the glandular epithelial cells, and the stroma immediately adjacent to the uterine lumen on Day 4.5, before the onset of implantation (Fig. 3a). Notably, this moderate but evident

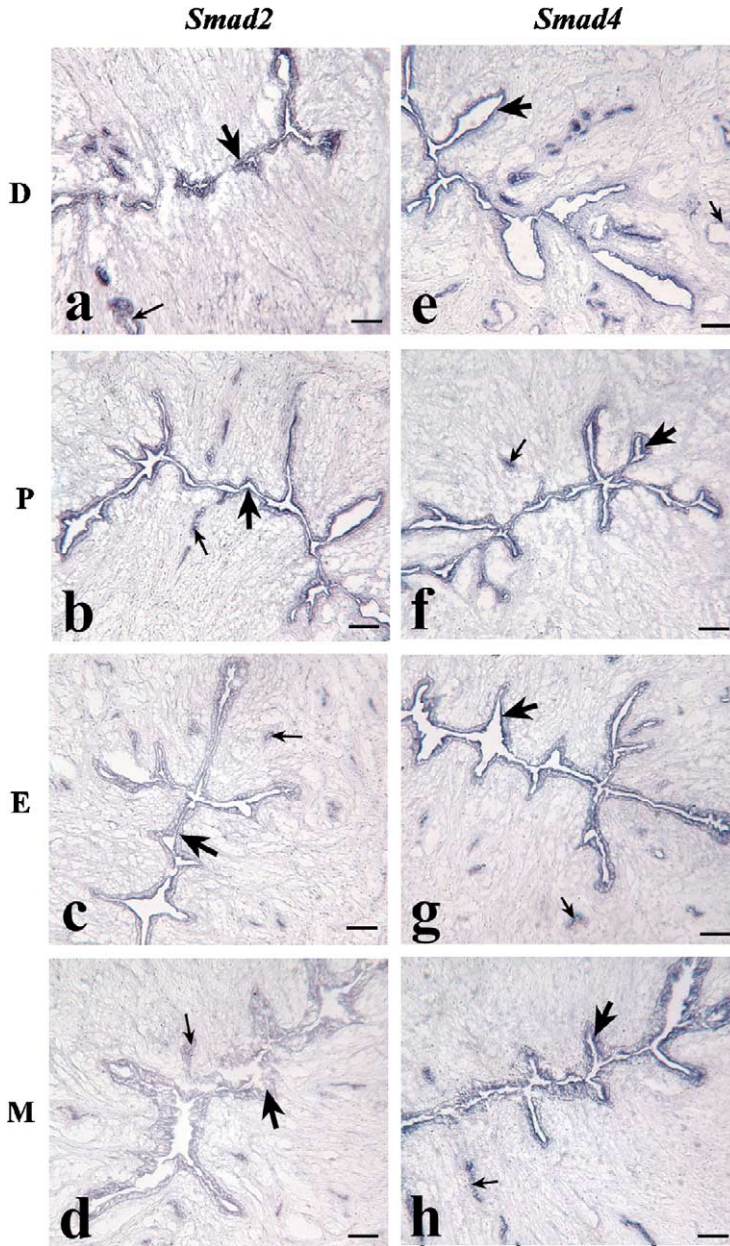


Fig. 1. In situ hybridization for *Smad2* and *Smad4* mRNAs in rat endometria during the estrous cycle. (a–d) *Smad2* expression in the cyclic endometria at diestrus, proestrus, estrus, and metaestrus, respectively. (e–h) *Smad4* expression in the cyclic endometria at diestrus, proestrus, estrus, and metaestrus, respectively. These results are representative of sections from three different rats. Thick arrow represents the luminal epithelium. Small arrow represents the glandular epithelium. Scale bar represents 200 μm .

Table 1

In situ hybridization for *Smad2* and *Smad4* mRNAs in different compartments of rat endometria at diestrus (D), proestrus (P), estrus (E), and metaestrus (M)

	<i>Smad2</i>				<i>Smad4</i>			
	D	P	E	M	D	P	E	M
Luminal epithelium	+++	+++	++	++	+++	+++	+++	+++
Glandular epithelium	+++	++	+	+	+++	++	++	++
Stroma	0	0	0	0	0	0	0	0
Blood vessel	0	0	0	0	0	0	0	0

The degree of expression were semiquantitated as high (+++), moderate (++), weak (+), and hardly no/no detectable staining (0).

expression detected in the luminal epithelium did not change substantially on Day 5.5, the time when the trophoblast invasion began, but an increase in the glandular epithelium and subepithelial stroma was noted (Fig. 3b). By Day 6.5 after implantation, moderate accumulation of *Smad2* mRNA occurred mainly in the luminal epithelium and the primary decidual zone. Signals in the secondary decidual zone and blastocyst were below the level of detection (Fig. 3c).

Different expression pattern for *Smad4* mRNA in the peri-implantation uteri was noted when compared with *Smad2* (Table 3). Dramatic staining for *Smad4* mRNA was found in the luminal epithelial cells, the glandular epithelial cells, and subepithelial stroma cells on Day 4.5, as shown in Fig. 3e. But the high expression levels in the luminal epithelium and subepithelial stroma were reduced to a moderate level on Day 5.5 (Fig. 3f). On Day 6.5, abundant accumulation of *Smad4* mRNA occurred primarily in the luminal epithelium and primary decidual zone surrounding the implanting blastocyst (Fig. 3g).

Signals were barely detected in the non-decidualized stroma (the stroma adjacent to the uterine lumen is not included) (Fig. 3 a, b, e, and f), blood vessel, and myometrium (data not shown) at the three time points of peri-implantation for both genes. Fig. 1d and h show representative negative control results of sense hybridization for *Smad2* and *Smad4* from the endometrium on Day 5.5, respectively.

Table 2

In situ hybridization for *Smad2* and *Smad4* mRNAs in different compartments of rat endometria on Days 0.5 (D0.5), 1.5 (D1.5), 2.5 (D2.5), and 3.5 (D3.5) of pregnancy

	<i>Smad2</i>				<i>Smad4</i>			
	D0.5	D1.5	D2.5	D3.5	D0.5	D1.5	D2.5	D3.5
Luminal epithelium	+++	++	+	++	+++	++	+++	+++
Glandular epithelium	+++	++	+	++	+++	+++	+++	+++
Stroma	0	0	0	0	0	0	0	0
Blood vessel	0	0	0	0	0	0	0	0

The degree of expression were semiquantitated as high (+++), moderate (++), weak (+), and hardly no/no detectable staining (0).

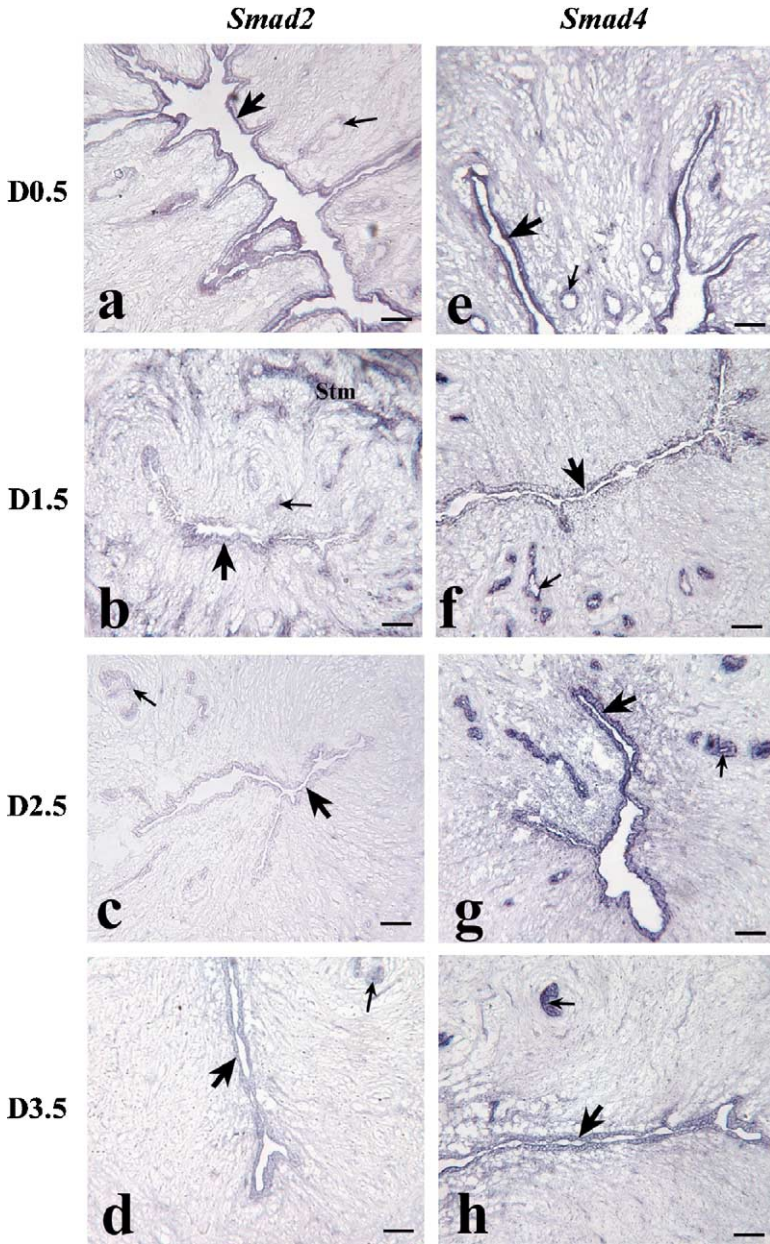


Fig. 2. In situ hybridization for *Smad2* and *Smad4* mRNAs in rat endometria during pre-implantation. (a–d) *Smad2* expression in the pregnant endometria on Days 0.5, 1.5, 2.5, and 3.5 of pregnancy, respectively. (e–h) *Smad4* expression in the pregnant endometria on Days 0.5, 1.5, 2.5, and 3.5 of pregnancy, respectively. These results are representative of sections from three different rats. Thick arrow represents the luminal epithelium. Small arrow represents the glandular epithelium. Scale bar represents 200 μ m. Stm: stromal cells adjacent to the myometrium.

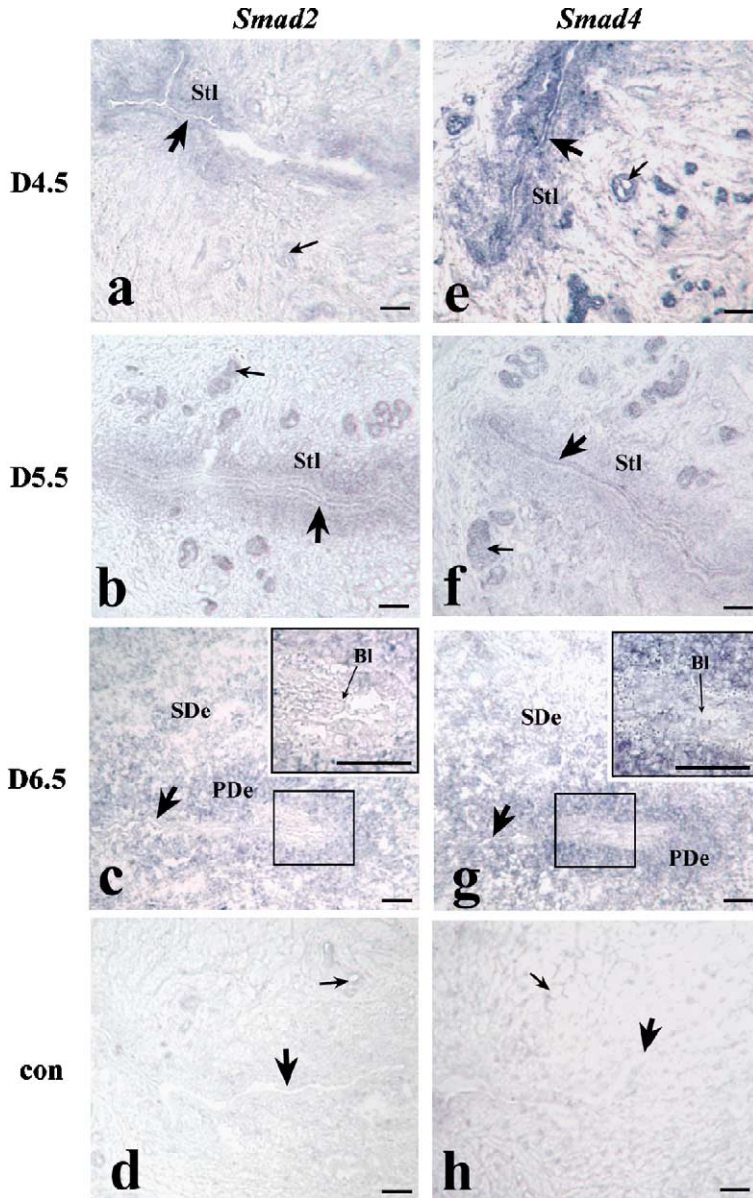


Fig. 3. In situ hybridization for *Smad2* and *Smad4* mRNAs in rat endometria during peri-implantation. (a–c) *Smad2* expression in the pregnant endometria on Days 4.5, 5.5, and 6.5 of pregnancy, respectively. (e–g) *Smad4* expression in the pregnant endometria on Days 4.5, 5.5, and 6.5 of pregnancy, respectively. (d and h) Negative controls of sense probes for *Smad2* and *Smad4* on Day 5.5 of pregnancy, respectively. These results are representative of sections from three different rats. Insets show higher power views of the boxed areas in (c) and (g), respectively. Thick arrow represents the luminal epithelium. Small arrow represents the glandular epithelium. Scale bar represents 200 μ m. Stl: stromal cells immediately adjacent to the uterine lumen; PDe: primary decidual zone; SDe: secondary decidual zone; BI: blastocyst.

Table 3

In situ hybridization for *Smad2* and *Smad4* mRNAs in different compartments of rat endometria on Days 4.5 (D4.5), 5.5 (D5.5), and 6.5 (D6.5) of pregnancy

	<i>Smad2</i>			<i>Smad4</i>		
	D4.5	D5.5	D6.5	D4.5	D5.5	D6.5
Luminal epithelium	++	++	++	+++	++	+++
Glandular epithelium	++	+++	/	+++	+++	/
Stroma adjacent to the lumen/primary decidual zone	++	+++	++	++++	++	+++
General stroma/decidua	0	0	0	0	0	0
Blood vessel	0	0	0	0	0	0

The degree of expression were semiquantitated as maximum (++++), high (+++), moderate (++) , weak (+), hardly no/no detectable staining (0), and unconvincing (/).

4. Discussion

The changing levels of ovarian steroids during the estrous/menstrual cycle cause the uteri to undergo extensive degrading and remodeling. The rat uterine endometrium proliferates rapidly corresponding to the high estrogen and progesterone levels at proestrus (Smith et al., 1975), and regresses following the loss of the proliferated cells if there is no embryo implantation (Parr and Parr, 1989). Our result, that *Smad2* was highly expressed in the luminal epithelium and the glandular epithelium at diestrus and proestrus while it was decreased at estrus and metaestrus, suggests that *Smad2* participates in the tissue degrading and restructuring of the cycling rat endometrium. Contrary to *Smad2*, the steady-state level of *Smad4* transcripts provides the possibility that SMAD4, as a common partner of all R-SMADs, may play a constitutive role in rat endometrium during the estrous cycle.

Expression of both *Smad2* and *Smad4* mRNAs during pre-implantation was highly compartmentalized and strictly programmed, with intense hybridization signals being confined to the luminal epithelium and the glandular epithelium. These results show some correspondence with previous findings on TGF- β s localization by Tamada et al. (1990) and Das et al. (1992), who found that TGF- β 1 mRNA and TGF- β 2 protein were expressed in the luminal and glandular epithelium of the mouse uterus on Days 1–4 of pregnancy. Moreover, the epithelial localization patterns of both *Smads* are also in line with TGF- β s localization profiles in species other than mice. For instance, TGF- β s localizations in these compartments were observed in ovine (Dore et al., 1996; Flores et al., 1998; Imakawa et al., 1998), equine (Lennard et al., 1995), and porcine (Gupta et al., 1998a, b) pregnant endometrium at mRNA and/or protein levels. Previous experiments have illustrated that TGF- β s could be produced by endometrial stroma (Tamada et al., 1990; Bruner et al., 1995), epithelium (Tamada et al., 1990; Das et al., 1992; Goffin et al., 2002), or other compartments such as myometrium and vascular smooth muscle (Das et al., 1992). Our findings, in conjunction with previous investigations, suggest that during pre-implantation period, TGF- β s function mainly in uterine epithelial cells in an autocrine fashion, and activate their signal transducers SMAD2 and SMAD4 to regulate their target genes primarily in these compartments. Regarding the role of epithelium in supporting the organization of endometrial stroma and myometrial differentiation, and the secretions of endometrial glands serving as primary

regulators of implantation and placentation (Gray et al., 2001), the epithelial localizations of TGF- β s, *Smad2*, and *Smad4* raise the possibility that TGF- β signaling is involved in the marked endometrial cell proliferation and architectural changes during pre-implantation.

Preovulatory secretion of ovarian estrogen in mouse results in the proliferation of the luminal epithelium and the glandular epithelium on Days 1 and 2 of pregnancy, respectively, in preparation to receive the implanting embryo (Tamada et al., 1990). Accordingly, the accumulations of *Smad2* and *Smad4* mRNAs in rat uterine epithelium on Days 0.5 and 1.5, as shown by our investigation, suggest that they are involved in epithelial cells proliferation. From Day 3 onwards the proliferation of the stromal cells and the differentiation of the luminal epithelium were started in response to the rising progesterone levels in mouse (Tamada et al., 1990). The data in this study presented that contrary to *Smad4* mRNA, which was abundantly expressed in the uterine epithelium on Day 2.5, *Smad2* mRNA expression was highly down-regulated. These results highlight the role of *Smad4* in modulating the initiation of the extensive stromal cell proliferation and luminal epithelial cell differentiation on Day 2.5 while de-emphasize the function of *Smad2* in this process. On Day 4 of rat pregnancy, the endometrium is in a neutral phase when the blastocyst enters into the endometrial cornu. It is quickly switched to the receptive phase on Day 5 of pregnancy (Tabibzadeh and Babaknia, 1995). Our findings, that there was an increase of *Smad2* expression level in the luminal epithelium and the glandular epithelium on Day 3.5, compared with that on Day 2.5, indicate that epithelial *Smad2* could contribute to the switch from the neutral phase to the receptive phase of the endometrium. The absence of *Smad2* and *Smad4* mRNAs in rat endometrial blood vessels during the pre-implantation period suggest that they are not involved in the endometrial vascular permeability, which is of general importance in the establishment of pregnancy (Abrahamsohn and Zorn, 1993). The expression of *Smad4* transcripts showed an overall higher and more steady level than that of *Smad2* during rat pre-implantation, as reported herein, raise the possibility regarding the more constitutive function of *Smad4* in the endometrium during rat pre-implantation period.

Successful embryo implantation requires both the invasion of the blastocyst trophoblast and the reception of the uterine endometrium for their future interaction. Days 4.5, 5.5, and 6.5 are the three critical time points that coincide with the three approaches of rat peri-implantation, the preparation of the attachment reaction, the commencement and accomplishment of the invasion of the trophoblast cells, respectively. Interestingly, gene expression for both *Smad2* and *Smad4* in the endometrium detected by in situ hybridization showed different distribution profiles from Day 4.5 onwards, compared with those during pre-implantation. Their expression was accumulated in the luminal epithelium and subepithelial stroma, the sites where attachment reaction and trophoblast invasion happen, indicating that both *Smads* serve as maternal regulators in these compartments of the receptive uterus to facilitate the dialogue between the embryo and the mother during implantation. The onset of the receptive phase of rat endometrium on Day 4.5 is characterized by the changes in the apical plasma membrane of the luminal epithelium (Tabibzadeh and Babaknia, 1995). The accumulation of *Smad4* mRNA in the luminal epithelium, the glandular epithelium, and subepithelial stromal cells on Day 4.5 pinpoints the role of *Smad4* during the establishment of the uterine receptivity. Quite different from *Smad2*, which maintained its moderate transcription level on Day 5.5, *Smad4* gene expression showed a decrease trend. Relative gene expression for *Smad2* contrasted with *Smad4* in that its total level was approximately

constant at the three time points of peri-implantation. In light of these observations, we conclude that *Smad2* plays constitutive functions, with fluctuation in local compartments of the endometrium. The interestingly down-modulated expression of *Smad4* in the luminal epithelium and the adjacent stroma, and the moderate expression of *Smad2*, just at the time when trophoblast invasion began, might support the hypothesis that TGF- β signaling was down-regulated in the process of trophoblast invasion. The localization of *Smad2* and *Smad4* mRNAs in the primary decidual zone on Day 6.5 of rat pregnancy, as observed herein, is consistent with the localization of activin β A on Day 5.5 of mouse pregnancy (Manova et al., 1992; Albano et al., 1994), but contrast with the secondary decidual zone localization of TGF- β 1 on Day 5.5 of mouse pregnancy (Tamada et al., 1990), indicating that *Smad2* and *Smad4* may be involved in the formation of the primary decidual zone together with activin at the time of the accomplishment of the trophoblast invasion in rat and mouse, but they may not play roles in mediating interactions between the primary decidual zone and the secondary decidual zone, a possibility presented by Tamada et al. (1990). The barely detectable expression of *Smad2* and *Smad4* in the blastocyst on Day 6.5, similar to the absence of TGF- β 2, TGF- β 3 (Das et al., 1992), and activin (Albano et al., 1994) in mouse blastocyst on Day 5.5, indicates that TGF- β s/activins may not serve as paracrine mediators for the trophoblast differentiation just after the accomplishment of implantation. However, the embryonic as well as the uterine localizations of TGF- β s (Manova et al., 1992; Chen et al., 1993; Cheng et al., 1993; Roelen et al., 1994) and activins (Manova et al., 1992, 1995; Albano et al., 1994) during mouse/rat post-implantation stages, suggest that TGF- β s/activins may act in autocrine or paracrine fashions in regulating the embryogenesis and placentation.

In conclusion, this study demonstrates that rat cycling, pre-, and peri-implantation endometria express *Smad2* and *Smad4* transcripts in different spatiotemporal patterns. The different expression profiles of *Smad2* and *Smad4* we found in the present study, have provided us a mechanistic framework for understanding the possibility that TGF- β s and activins may be involved in different aspects of the estrous cycle and implantation. An investigation of the expression patterns of different *Smads* in various species, coupled to finding more target DNA sequences, might provide more detailed information on the molecular mechanism of TGF- β regulation during the estrous cycle and implantation.

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