Expression of *Smad2* and *Smad4* in rhesus monkey endometrium during the menstrual cycle and early pregnancy*

LIN Haiyan[1], WANG Hongmei[1], LI Qinglei[1], WANG Juan[2], ZHANG Xuan[1], LIU Donglin[1], LIU Huitu[2] and ZHU Cheng[1]**

(1. State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100080, China; 2. Key Laboratory for Cell Proliferation and Regulation of Ministry of Education, Institute of Cell Biology, College of Life Sciences, Beijing Normal University, Beijing 100875, China)

Received December 9, 2002; revised February 17, 2003

Abstract Expression of *Smad2* and *Smad4* mRNAs in the endometrium of rhesus monkey on Days 8, 20 and 28 of the normal menstrual cycle and on Days 12, 18 and 26 of early pregnancy was detected using *in situ* hybridization. The results showed that *Smad2* and *Smad4* mRNAs were mainly localized in luminal epithelium and glandular epithelium. The expression of *Smad2* mRNA in glandular epithelium was sustained at moderate level on Days 8, 20 and 28 of the menstrual cycle, while the expression of *Smad4* gradually increased with the menstrual cycle. Both *Smad2* and *Smad4* mRNAs in functionalis glandular epithelium were expressed at the highest levels on Day 12 of early pregnancy, while in basalis glandular epithelium the most abundant expression of both *Smads* occurred on Days 12 and 18 of pregnancy. On Day 26, both *Smads* mRNAs were expressed at the lowest levels either in functionalis or in basalis. The data suggest that the epithelium is the major compartment where TGF-βs/activins exert their biological effects via *Smads*, and that *Smad4* may play a role in the maintenance of endometrial gland function during secreting period of the menstrual cycle. During lacunar stage of early pregnancy, *Smad2* and *Smad4* are implicated in the tissue remodeling of endometrial functionalis and basalis, and during early villous stage both *Smads* are functional primarily in basalis.

Keywords: Smad, rhesus monkey, pregnancy, menstrual cycle, endometrium.

The transforming growth factor-β (TGF-β) superfamily consisting of TGF-βs, activins, bone morphogenic proteins (BMPs), etc. is a multifunctional polypeptide superfamily that functions in many biological events such as cell proliferation and differentiation, embryo development, angiogenesis and extracellular matrix remodeling[1]. Smad proteins were discovered as intracellular signaling molecules that transmit the extracellular TGF-β signals from the transmembrane serine/threonine kinase receptors to the nucleus where they participate in the activation of target genes. To date, Smads, constituting a family of nine members in vertebrates, have been classified into three subfamilies. Upon ligand binding, TGF-βR Ⅱ (TGF-β type Ⅱ receptor) and TGF-βR Ⅰ (TGF-β type Ⅰ receptor) form heteromers, and subsequently phosphorylate the downstream mediators R-Smads (receptor-regulated Smads), of which Smad1, Smad5 and Smad8 respond to BMPs whereas Smad2 and Smad3 mediate TGF-βs/activins signals. R-Smads then form heteromeric complexes with Co-Smads (common-partner Smads) (Smad4 and Smad4β in *Xenopus*), followed by the translocation of the complexes into the nucleus. Inhibitory Smads (I-Smads), as a third subfamily, include Smad6 and Smad7 which function to inhibit the TGF-β signaling cascade[2]. In some cases, TGF-βs could activate the mitogen-activated protein kinase (MAPK) pathway or PP2A/p70s6k pathway independent of Smads[3]. Smads, however, were still believed to be the primary mediators of TGF-β biological effects.

The highly dynamic and cyclic primate endometrium possesses regular cycles of growth, differentiation and degradation, which is essential for the sustentation of normal menstrual cycle and successful embryo implantation. There was evidence to prove that TGF-βs were expressed together with their receptors in human fetal-maternal interface[4], endometrium[5] and pre-implantation embryo[6]. TGF-β can also regulate the expression of trophoblastic[7~10] and endometrial[11~13] matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs). Thus, TGF-β may be involved in the regulation of

* Supported by the Special Funds for Major State Basic Research Project (Grant No. G1999055903) and CAS Innovation Program (Grant No. KSCX3-IOZ-07)

** To whom correspondence should be addressed. E-mail: zhuc@panda.ioz.ac.cn

primate endometrial modification in an autocrine and paracrine fashion. Human endometrium compartments also express activin during the menstrual cycle and embryo implantation[14~16]. However, few reports have clearly demonstrated the presence of Smads in the endometrium, despite their important roles in mediating TGF-βs/activins actions. Since the accomplishment of biological actions of TGF-βs/activins is determined by the activation of the receptors as well as intracellular signaling of Smads, neither the specific localization of TGF-βs/activins extracellular ligands nor their receptors could provide enough evidence for their function. In light of this consideration, the investigation of the localization of Smads is essential to determining whether and where TGF-βs/activins function.

To explore whether TGF-βs/activins signaling is operational in primate uterus during the menstrual cycle and early pregnancy, we investigated the expression patterns of *Smad2* and *Smad4*, as R-Smad and Co-Smad in TGF-βs/activins signaling cascades respectively, in rhesus monkey endometrium using *in situ* hybridization.

1 Materials and methods

1.1 Animals and tissue collection

The uteri of 12 pregnant rhesus monkeys (*Macaca mulatta*) were collected in the Center for Medical Primate, Institute of Medical Biology, Chinese Academy of Medical Sciences as described previously[17]. In brief, adult female rhesus monkeys with a history of regular menstrual cycles and pregnancies were allowed to mate with the males for two days from the anticipated time of ovulation. Sperm plug examination, ultrasonography and mCG detection in the uterus were performed to determine the pregnancy. The anticipated day of ovulation was designated as Day 0 of pregnancy. On Days 8 ($n=2$), 20 ($n=2$) and 28 ($n=2$) of the normal menstrual cycle, and on estimated Days 12 ($n=2$), 18 ($n=2$) and 26 ($n=2$) of pregnancy, monkeys were laparotomized under ketamine hydrochloride anaesthesia. Uteri were removed and trimmed laterally. The implantation sites were embedded in embedding medium (Triangle Biomedical Sciences, USA) and stored at −80 ℃ until analyzed.

1.2 Immunohistochemistry

A biotin-avidin-peroxidase complex (ABC) method (Vectastain ABC kit, Vector Labs., USA) was used for detection of cytokeratin in rhesus monkey uteri during normal menstrual cycle or early pregnancy. A rabbit anti-cytokeratin antibody (Santa Cruz Biotechnology Inc., USA) and the biotinylated anti-rabbit IgG (Vector Labs.) were used in immunohistochemistry. The sections were counterstained with Harris hematoxylin and mounted for photography.

1.3 Subcloning of *Smad2* and *Smad4*

C-Flag-tagged human *Smad2* and *Smad4* mammalian expression plasmids[18] were the 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, USA), respectively. The full-length *Smad2* and *Smad4* cDNA fragments were obtained by digesting the two plasmids with *Eco*R Ⅰ and *Hin*d Ⅲ. After being purified from the agarose gel using NucleoTrap Gel Extraction Kit (CLONTECH Laboratories Inc., USA), the two fragments were then subcloned into vector pGEM-3Z (Promega, USA).

1.4 Probe labeling and concentration determination

To generate antisense RNA probes, the plasmids were linearized with *Eco*R Ⅰ and transcribed with SP6 RNA polymerase using digoxigenin (DIG) RNA labeling kit (SP6/T7) (Boehringer-Mannheim, USA). Sense RNA probes were synthesized with *Hin*d Ⅲ and T7 RNA polymerase. The yields of the probes were determined using spot test following the manufacturer's instructions with a DIG-labeled RNA control provided in the labeling kit. Spot intensities of the control and experimental RNA probes were quantified using Band Leader version 3.0 (Magnitec Ltd., Israel) to estimate the concentration of the experimental probes.

1.5 *In situ* hybridization

Adjacent cryostat sections (10 μm) were fixed in 4% paraformaldehyde, incubated in PBS containing 0.1% active DEPC for 30 min, and then incubated in 5 × SSC for 15 min at room temperature. Prehybridization was carried out at 50 ℃ for 2 h in a buffer containing 50% deionized formamide, 5 × SSC and 120 μg/mL salmon sperm DNA. Slides were then hybridized with 400 ng/mL of the labeled probe overnight at 50 ℃. In case of nonspecific binding,

the slides were serially washed as follows: 2 × SSC at room temperature for 30 min, 2 × SSC at 65 ℃ for 1 h, 0.1 × SSC at 65 ℃ for 1 h, buffer A (100 mmol/L Tris, 150 mmol/L NaCl, pH 7.5) at room temperature for 5 min. This was followed by incubation with anti-digoxigenin-alkaline phosphatase for 2 h. After washing with buffer A, the slides were incubated in buffer C (100 mmol/L Tris, 100 mmol/L NaCl, 50 mmol/L $MgCl_2$, pH 9.5) for 5 min, and the color was developed by nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Boehringer-Mannheim). All the experiments were repeated three times. Sense probe hybridization was used as a control for the background level, and the results were analyzed by SPOT digital image system (Diagnostic Instruments Inc., USA).

1.6 Statistical analysis

Signal intensities of *Smad2* and *Smad4* mRNAs in glandular epithelia detected by *in situ* hybridization were determined by computer-aided laser scanning densitometry (Personal Densitometer SI, Molecular Dynamics Inc., USA). In order to make the statistical significance of the quantitative difference credible in each uterine sample, 12 spots were randomly selected in glandular epithelia in the experimental set, and in the corresponding glandular epithelial compartments in the control set. Values are corrected sample means ± SEM. Statistical analysis was performed using Statistical Package for Social Science (SPSS for Windows package release 10.0, SPSS Inc., USA). One-way ANOVA was used in evaluating the difference between individual groups of normal menstrual cycle or early pregnancy. $P<0.05$ and $P<0.01$ were considered to be statistically significant. Data of glandular epithelium in the functionalis zone (GEf) and in the basalis zone (GEb) were analyzed for statistical differences with Student's *t*-test to verify differences between individual groups.

2 Results

2.1 Immunohistochemical staining of cytokeratin in rhesus monkey endometrium

The typical structure of the macaque endometrium was confirmed by immunohistochemical staining of cytokeratin, with Harris hematoxylin counterstaining. Luminal epithelium and glandular epithelium were cytokeratin-positive. Cytotrophoblast and syncytiotrophoblast lining the villi, fetal-maternal interface, stroma, arteriole and myometrium were cytokeratin-negative. Plate Ⅰ (a)~(c) shows the representative structure of glandular epithelium and stroma on Days 8, 20 and 28 of normal menstrual cycle, respectively. The typical structure of luminal epithelium, glandular epithelium and stroma on Days 12, 18 and 26 of early pregnancy is shown in Plate Ⅱ (a)~(f).

2.2 *Smad2* and *Smad4* mRNA expressions in rhesus monkey endometrium during the menstrual cycle

Smad2 and *Smad4* mRNA expressions were observed mainly in glandular epithelium of the macaque endometrium on Days 8, 20 and 28 of the menstrual cycle (Plate Ⅰ (d)~(i)). Expression of both *Smads* in stroma was relatively low. Both *Smads* in arteriole and myometrium were undetectable. Sense-strand probes for both genes showed no specific hybridization signals in any endometrium investigated.

Smad2 mRNA expression in glandular epithelium remained at moderate level on Days 8, 20 and 28 (Plate Ⅰ (d)~(f)), with no discernable change (Fig. 1(a)). The overall expression level of *Smad4* in glandular epithelium was higher than that of *Smad2*. In addition, *Smad4* expression increased gradually from Day 8, through Day 20, to Day 28, with a pattern distinct from that of *Smad2* (Plate Ⅰ (g)~(i)). The expression levels between individual groups were significantly different (Fig. 1(b)).

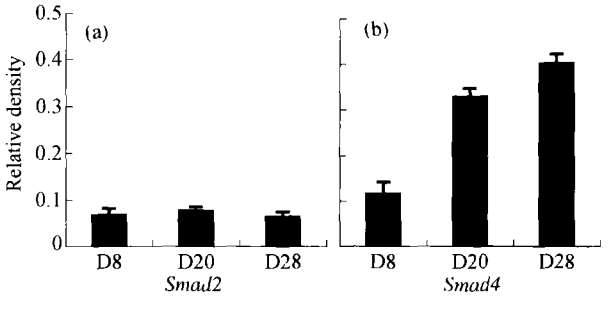

Fig. 1. Graphic representation of the mean relative expression levels of *Smad2* and *Smad4* mRNAs in glandular epithelium of rhesus monkey endometrium on Days 8 (D8), 20 (D20) and 28 (D28) of normal menstrual cycle. Means of relative densities were calculated from the computer-assisted densitometric scan. Error bars are SEM calculated from the corrected sample means to indicate sample variation within each day of menstrual cycle. (a) Relative expression levels of *Smad2*. No significant difference exists among those groups. (b) Relative expression levels of *Smad4*. The difference between time points D20 and D28 is significant ($P<0.05$), and the differences between time points D8 and D20/D28 are significant ($P<0.01$).

2.3 *Smad2* and *Smad4* mRNA expressions in rhesus monkey endometrium during early pregnancy

Smad2 and *Smad4* mRNAs were expressed mainly in luminal epithelium and glandular epithelium on Day 12 of pregnancy, and in glandular epithelium on Days 18 and 26 of pregnancy. The expression of *Smad2* and *Smad4* in decidual cells was relatively low. No expression of *Smad2* or *Smad4* was detected in arteriole or myometrium (Plate Ⅱ (h), (i), (k), (l), (n), (r)). Staining in trophoblast shell as well as fetal-maternal interface was also below the level of detection (data not shown). Expression of *Smad2* and *Smad4* mRNAs in glandular epithelium changed with the progress of pregnancy, in cell-specific patterns. And their expression patterns in functionalis epithelial cells were different from those in basalis epithelial cells (Plate Ⅱ (g)～(r)). Positive *in situ* hybridization signal for *Smad2* mRNA in glandular epithelium located in functionalis was significantly decreased with the progress of pregnancy (Fig. 2 (a) and Plate Ⅱ (g), (i), (k)). Expression of *Smad2* mRNA in glandular epithelium located in basalis was different from that in functionalis. *Smad2* mRNA was detected at a high level on Day 12 of pregnancy (Plate Ⅱ (h)). Its expression on Day 18 significantly increased and reached the highest level (Fig. 2 (a) and Plate Ⅱ (j)), but decreased dramatically on Day 26 of pregnancy (Fig. 2 (a) and Plate Ⅱ (l)).

Expression of *Smad4* mRNA in the endometrial compartments showed a distinct manner, and was much higher than that of *Smad2* mRNA. In functionalis glandular epithelium, expression of *Smad4* mRNA on Day 12 was the highest (Plate Ⅱ (m)). Its expression on Day 18 was significantly decreased (Fig. 2(b) and Plate Ⅱ (o)), and sustained on Day 26 (Plate Ⅱ (q)). In basalis glandular epithelium, the highest expression level of *Smad4* was on Day 12 and Day 18, but the expression on Day 26 was significantly decreased (Fig. 2 (b) and Plate Ⅱ (n), (p), (r)).

Expression difference of *Smad2* mRNA in glandular epithelium between functionalis and basalis coincided with that of *Smad4*. On Day 12, expression of both *Smad2* and *Smad4* in functionalis glandular epithelium was significantly higher than that in basalis glandular epithelium (Fig. 2 and Plate Ⅱ (g), (h), (m), (n)). While on Day 18, positive *in situ* hybridization signal for *Smad2* and *Smad4* in basalis glandular epithelium was strikingly higher than that in functionalis glandular epithelium (Fig. 2 and Plate Ⅱ (i), (j), (o), (p)). On Day 26, there was no significant difference in the expression of *Smad2* and *Smad4* mRNAs between functionalis glandular epithelium and basalis glandular epithelium (Fig. 2 and Plate Ⅱ (k), (l), (q), (r)).

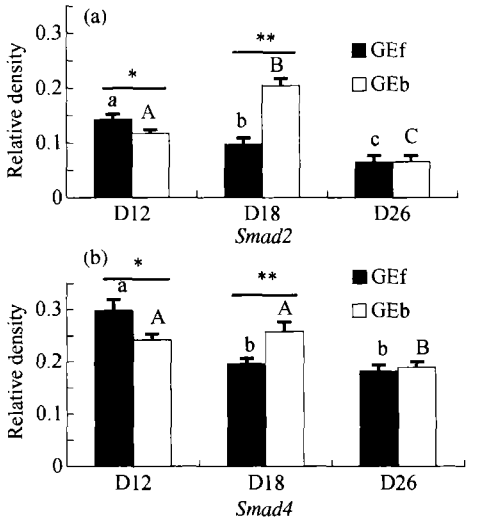

Fig. 2. Graphic representation of the mean relative expression levels of *Smad2* and *Smad4* mRNAs in glandular epithelium of rhesus monkey endometrium on Days 12 (D12), 18 (D18) and 26 (D26) of pregnancy. Means of relative densities were calculated from the computer-assisted densitometric scan. Error bars are SEM calculated from the corrected sample means to indicate sample variation within each day of early pregnancy. (a) Relative expression levels of *Smad2*. Time points with no characters in common are significantly different at $P<0.05$ or at $P<0.01$, with a, b and c representing expression differences in glandular epithelium of the functionalis (GEf), and A, B and C representing expression differences in glandular epithelium of the basalis (GEb). Among those, the expression difference in the GEf between time points D18 and D26 is significant ($P<0.05$), and the differences between time points D12 and D18/D26 are significant ($P<0.01$). The expression differences in the GEb among time points D12, D18 and D26 are all significant ($P<0.01$). (b) Relative expression levels of *Smad4*. a and b on top of each time point represent significant differences ($P<0.01$) in the expression levels in GEf between time points D12 and D18/D26, and no significant difference between time points D18 and D26. A and B on top of each time point represent significant differences ($P<0.05$) in the expression levels in GEb between time points D12/D18 and D26, but no significant difference between time points D12 and D18. * and * * represent the significant difference in the expression levels between GEf and GEb at $P<0.05$ on D12 and at $P<0.01$ on D18, respectively. No significant difference exists between GEf and GEb on D26.

3 Discussion

This study has provided the first evidence that *Smad2* and *Smad4* mRNAs are expressed in rhesus monkey endometrium, thus supporting the hypothesis

that TGF-βs/activins signaling pathway is operational in macaque endometrium during the menstrual cycle and early pregnancy.

It has been demonstrated that TGF-β participates in endometrial tissue remodeling via inhibiting epithelial cell proliferation and differentiation[19] and maintaining the balanced expression of MMPs/TIMPs[8]. Secretions of endometrial glands function as primary regulators of embryogenesis, embryo implantation and placentation. Interaction between glandular epithelium and stroma will lead to local control and coordination of morphogenetically important cell behaviors such as movement, adhesion, differentiation and proliferation[20]. Strong evidence has documented the epithelial localization of TGF-βs[5,21~23] and activin[14~16,24] in the cyclic and pregnant endometria in many species. Combined with these reports, the limited localization of *Smad2* and *Smad4* in luminal epithelium and glandular epithelium, as shown in our results, may help us understand that the endometrial epithelium is the primary compartment of operational TGF-β/activins signaling. It was generally recognized that growth factors are secreted from stromal cells[20]. Stromal-derived paracrine TGF-β was found necessary for suppression of epithelial MMPs expression[11]. In contrast, regulation of stromal MMP-2 expression and secretion involves paracrine TGF-β derived from the epithelial compartments[13]. In light of these observations, it can be concluded that stromal-derived or epithelial-derived TGF-β may act in an autocrine and/or paracrine manner to modulate endometrial function during the menstrual cycle and early pregnancy, but the epithelium is the major compartment where TGF-β exerts its biological effect. In addition, the new results may suggest that TGF-βs/activins function to regulate target gene transcriptions via epithelial *Smad2* and *Smad4*, thus maintaining the normal function of epithelial cells during the menstrual cycle and pregnancy.

In women and menstruating primates, the menstrual cycle is subdivided into proliferating period, secreting period and menstrual period, during which the endometrium undergoes cyclical degradation and regeneration. Days 8, 20 and 28 correspond to proliferating period, early secreting period and late secreting period of the macaque menstrual cycle respectively. In this study, we showed that *Smad2* remained at a moderate level in the endometrial gland on Days 8, 20 and 28 of macaque menstrual cycle, suggesting its constitutive role in the normal menstrual cycle. In contrast, *Smad4*, as a common partner of R-Smads, displayed a gradually increased expression pattern on Days 8, 20 and 28, with the progress of the menstrual cycle. This intensive expression profile during the secreting period corresponds to the high expression trends of TGF-β[5,25] and activin[14,15], thus indicating that *Smad4* may play a role in the maintenance of endometrial gland function during secreting period of the menstrual cycle.

In rhesus monkey, the embryo implantation occurs on Day 9.5 of pregnancy[26]. The selected uteri on Days 12, 18 and 26 of pregnancy in our study represent tissues from lacunar stage, early villous stage and villous stage during the developmental process of the placenta respectively[27,28]. The data presented in this study showed that the most intensive expression of *Smad2* and *Smad4* in functionalis glandular epithelium occurred on Day 12, while in basalis the most abundant expression of both *Smads* occurred on Days 12 and 18. On Day 26, both *Smads* were expressed at the lowest levels either in functionalis or in basalis. More compelling was the observation that gene expression in glandular epithelium for both *Smads* showed different levels in functionalis and basalis, with significantly higher levels in functionalis than in basalis on Day 12, and significantly higher levels in basalis than in functionalis on Day 18. Taken together, these findings suggest that during lacunar stage, *Smad2* and *Smad4* in functionalis and basalis (primarily in functionalis) may serve to participate in the establishment of uterine epithelial morphology, which in turn contributes to the tissue remodeling of the endometrium, the early development of the villus and the invasion of the trophoblast cells. During early villous stage, *Smad2* and *Smad4* are functional primarily in basalis, which could be explained by the possibility that both *Smads* are implicated in the preparation of the receptive endometrium for the full invasion of the trophoblast cells into endometrial basalis. Although it has been reported that TGF-β was expressed in arterioles in Day 6 macaque endometrium[29], and that TGF-β or activin was localized in human first trimester fetal-maternal interface[4] and trophoblast[8,30,31], our data did not illustrate the localization of both *Smads* in these compartments at the three time points of early pregnancy, hinting that TGF-βs/activins signaling is not operational in primate endometrial arterioles, villi or fetal-maternal interface from Day 12 to Day 26 of pregnancy.

In conclusion, this study provides the direct evidence for the spatiotemporal expression of *Smad2* and *Smad4* in cyclic and pregnant macaque endometrium, and offers the potential to advance our understanding of operational TGF-β signaling during embryo implantation.

References

1 Massague, J. TGF-β signal transduction. Annu. Rev. Biochem., 1998, 67: 753.

2 Wrana, J. L. et al. The Smad pathway. Cytokine Growth Factor Rev., 2000, 11(1-2): 5.

3 Wakefield, L. M. et al. TGF-β signaling: positive and negative effects on tumorigenesis. Curr. Opin. Genet. Dev., 2002, 12(1): 22.

4 Graham, C. H. et al. Localization of transforming growth factor-β at the human fetal-maternal interface: role in trophoblast growth and differentiation. Biol. Reprod., 1992, 46(4): 561.

5 Chegini, N. et al. Human uterine tissue throughout the menstrual cycle expresses transforming growth factor-β1 (TGFβ1), TGFβ2, TGFβ3, and TGFβ type Ⅱ receptor messenger ribonucleic acid and protein and contains [125I] TGFβ1-binding sites. Endocrinology, 1994, 135(1): 439.

6 Osterlund, C. et al. TGFβ receptor types Ⅰ and Ⅱ and the substrate proteins Smad 2 and 3 are present in human oocytes. Mol. Hum. Reprod., 2000, 6(6): 498.

7 Graham, C. H. et al. Mechanism of control of trophoblast invasion *in situ*. J. Cell. Physiol., 1991, 148(2): 228.

8 Graham, C. H. et al. Mechanisms of placental invasion of the uterus and their control. Biochem. Cell Biol., 1992, 70(10-11): 867.

9 Shimonovitz, S. et al. Cytokine-mediated regulation of type IV collagenase expression and production in human trophoblast cells. J. Clin. Endocrinol. Metab., 1996, 81(8): 3091.

10 Meisser, A. et al. Effects of tumour necrosis factor-α, interleukin-1α, macrophage colony stimulating factor and transforming growth factor β on trophoblastic matrix metalloproteinases. Mol. Hum. Reprod., 1999, 5(3): 252.

11 Bruner, K. L. et al. Transforming growth factor β mediates the progesterone suppression of an epithelial metalloproteinase by adjacent stroma in the human endometrium. Proc. Natl. Acad. Sci. USA, 1995, 92(16): 7362.

12 Huang, H. Y. et al. Cytokine-mediated regulation of 92-kilodalton type IV collagenase, tissue inhibitor or metalloproteinase-1 (TIMP-1), and TIMP-3 messenger ribonucleic acid expression in human endometrial stromal cells. J. Clin. Endocrinol. Metab., 1998, 83(5): 1721.

13 Goffin, F. et al. Human endometrial epithelial cells modulate the activation of gelatinase a by stromal cells. Gynecol. Obstet. Invest., 2002, 53(2): 105.

14 Leung, P. H. et al. Immunolocalization of inhibin and activin subunits in human endometrium across the menstrual cycle. Hum. Reprod., 1998, 13(12): 3469.

15 Otani, T. et al. Immunohistochemical localization of activin A in human endometrial tissues during the menstrual cycle and in early pregnancy. Obstet. Gynecol., 1998, 91(5 Pt 1): 685.

16 Jones, R. L. et al. Inhibin and activin subunits are differentially expressed in endometrial cells and leukocytes during the menstrual cycle, in early pregnancy and in women using progestin-only contraception. Mol. Hum. Reprod., 2000, 6(12): 1107.

17 Wang, H. et al. Expression of matrix metalloproteinase-2, -9, -14, and tissue inhibitors of metalloproteinase-1, -2, -3 in the endometrium and placenta of rhesus monkey (Macaca mulatta) during early pregnancy. Biol. Reprod., 2001, 65(1): 31.

18 Zhang, Y. et al. Receptor-associated Mad homologues synergize as effectors of the TGF-β response. Nature, 1996, 383(6596): 168.

19 Moses, H. L. TGF-β regulation of epithelial cell proliferation. Mol. Reprod. Dev., 1992, 32(2): 179.

20 Gray, C. A. et al. Developmental biology of uterine glands. Biol. Reprod., 2001, 65(5): 1311.

21 Tamada, H. et al. Cell type-specific expression of transforming growth factor-β1 in the mouse uterus during the periimplantation period. Mol. Endocrinol., 1990, 4(7): 965.

22 Das, S. K. et al. Expression of transforming growth factor-β isoforms (β2 and β3) in the mouse uterus: analysis of the peri-implantation period and effects of ovarian steroids. Endocrinology, 1992, 130(6): 3459.

23 Lennard, S. N. et al. Transforming growth factor β1 expression in the endometrium of the mare during placentation. Mol. Reprod. Dev., 1995, 42(2): 131.

24 Yamanouchi, K. et al. Equine inhibin/activin β A-subunit mRNA is expressed in the endometrial gland, but not in the trophoblast, during pregnancy. Mol. Reprod. Dev., 1997, 47(4): 363.

25 Kauma, S. et al. Interleukin-1β, human leukocyte antigen HLA-DRα, and transforming growth factor-β expression in endometrium, placenta, and placental membranes. Am. J. Obstet. Gynecol., 1990, 163(5 Pt 1): 1430.

26 Enders, A. C. et al. Implantation in the rhesus monkey: initial penetration of endometrium. Am. J. Anat., 1983, 167(3): 275.

27 Ghosh, D. et al. Expression of vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) in conceptus and endometrium during implantation in the rhesus monkey. Mol. Hum. Reprod., 2000, 6(10): 935.

28 Carson, D. D. et al. Embryo implantation. Dev. Biol., 2000, 223(2): 217.

29 Ghosh, D. et al. Effect of early luteal phase administration of mifepristone (RU486) on leukaemia inhibitory factor, transforming growth factor β and vascular endothelial growth factor in the implantation stage endometrium of the rhesus monkey. J. Endocrinol., 1998, 157(1): 115.

30 Minami, S. et al. Immunohistochemical localization of inhibin/activin subunits in human placenta. Obstet. Gynecol., 1992, 80(3 Pt 1): 410.

31 Caniggia, I. et al. Activin is a local regulator of human cytotrophoblast cell differentiation. Endocrinology, 1997, 138(9): 3976.

LIN, Haiyan. et al. Expression of *Smad2* and *Smad4* in rhesus monkey endometrium during the menstrual cycle and early pregnancy

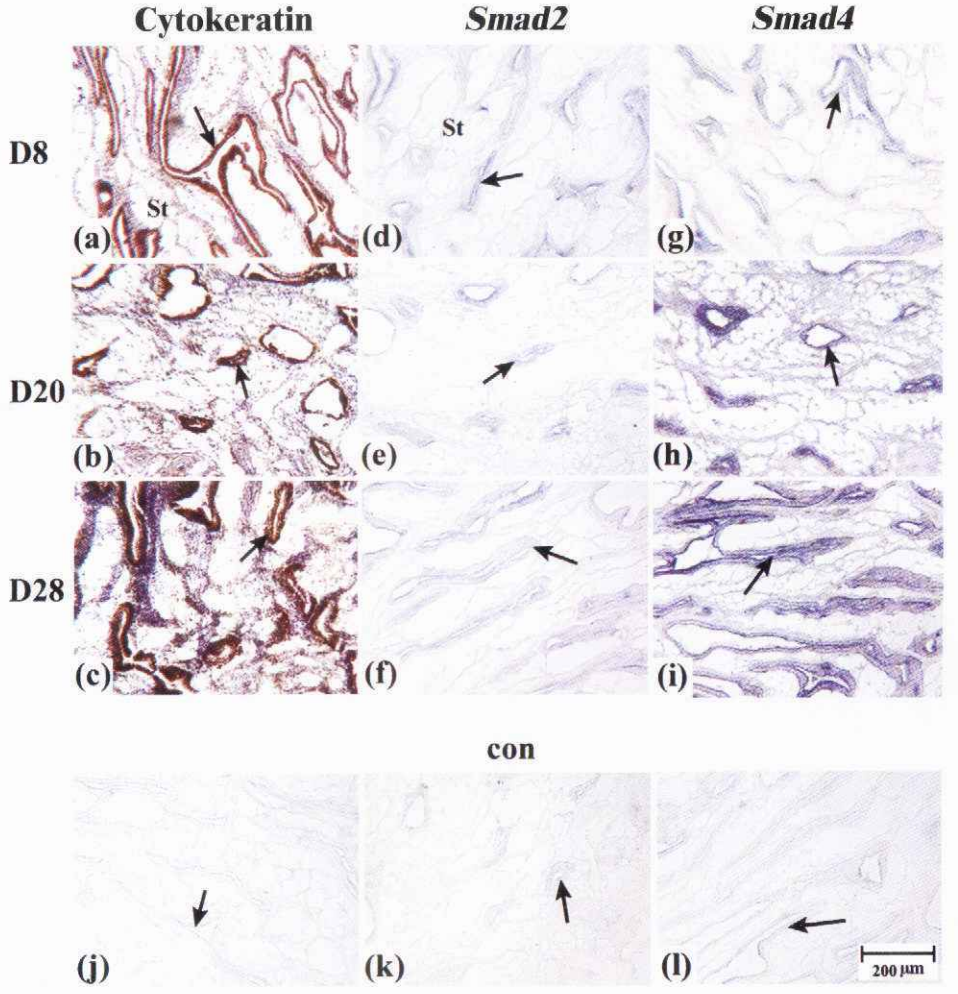

Plate Ⅰ. Expression of *Smad2* and *Smad4* mRNAs in rhesus monkey endometrium during normal menstrual cycle. (a)~(c) shows immunolocalization of cytokeratin in the endometrium of rhesus monkey on Days 8 (D8), 20 (D20) and 28 (D28) of normal menstrual cycle, respectively, with hematoxylin counterstaining. Note that positive cytokeratin staining is present in glandular epithelium. (d)~(f) shows the expression of *Smad2* mRNA in the endometrium on D8, D20 and D28, respectively. (g), (h) and (i) show the expression of *Smad4* mRNA in the endometrium on D8, D20 and D28, respectively. Representative negative controls of sense probes are shown in (j), (k) and (l), corresponding to the *in situ* hybridization results using *Smad2* sense probe in the endometrium on D8, D20 and D28, respectively. All the pictures are of identical magnification as shown in (l). Arrows show glandular epithelium (GE). (Con, control; St, stroma.)

LIN, Haiyan. et al. Expression of *Smad2* and *Smad4* in rhesus monkey endometrium during the menstrual cycle and early pregnancy

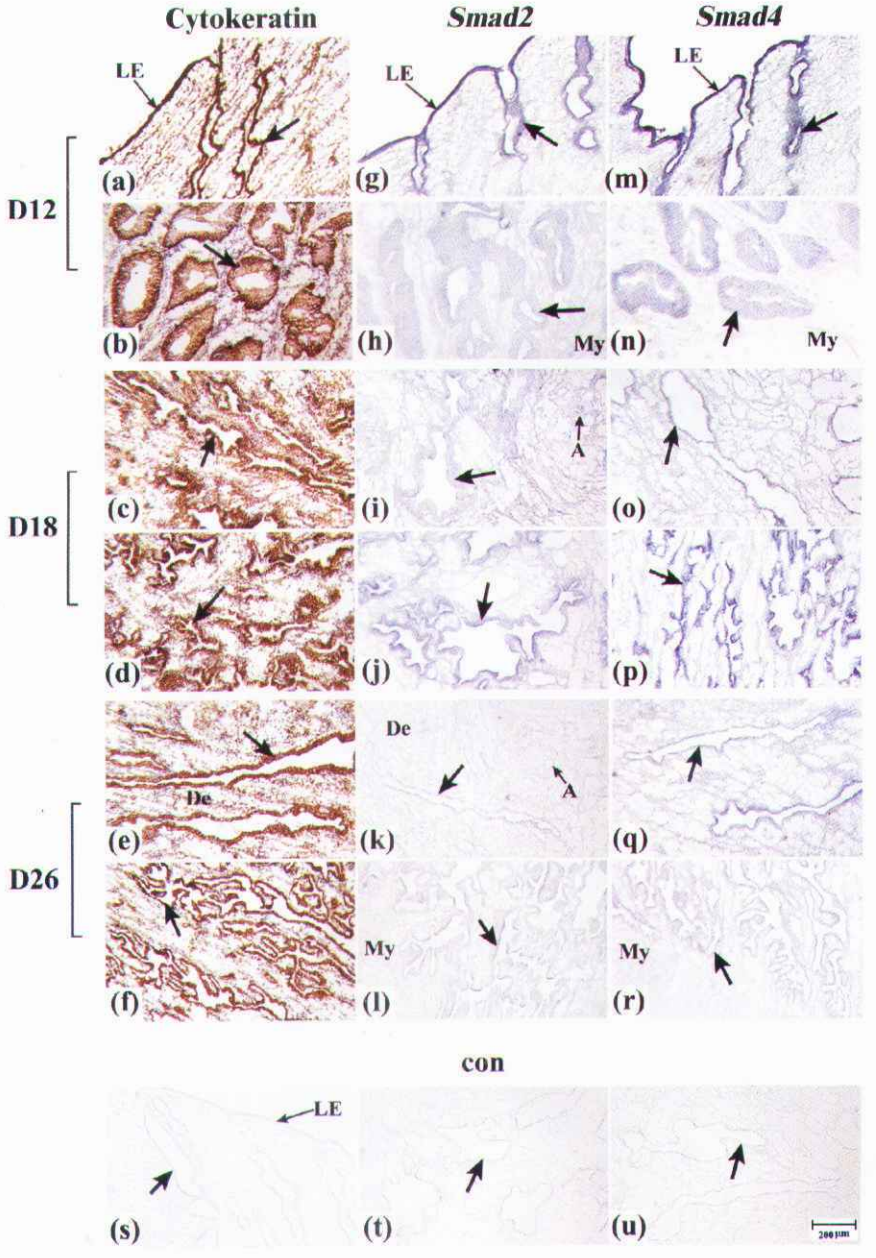

Plate Ⅱ. Expression of *Smad2* and *Smad4* mRNAs in rhesus monkey endometrium during early pregnancy. Cytokeratin immunostaining in the endometrium on Days 12 (D12), 18 (D18) and 26 (D26) of pregnancy, with hematoxylin counterstaining, are shown in (a)~(f). Note that positive cytokeratin staining is present in luminal epithelium and glandular epithelium. (g)~(l) show the expression of *Smad2* mRNA in the endometrium of rhesus monkey on D12, D18 and D26 of early pregnancy, respectively. (m)~(r) show the expression of *Smad4* mRNA in the endometrium of rhesus monkey on D12, D18 and D26 of early pregnancy, respectively. Control sections of endometrial functionalis on D12 (s), D18 (t) and D26 (u) hybridized to the *Smad2* sense probe show absence of the specific signals. All the pictures are of identical magnification as shown in (u). Thick arrows show glandular epithelium (GE). (Con, control; LE, luminal epithelium; De, decidua; A, arteriole; My, myometrium.)