Activation of Uterine Smad3 Pathway Is Crucial for Embryo Implantation*

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Summary: Embryo implantation is a complicated physiological process tightly regulated by multiple biological molecules including growth factors. Transforming growth factor-betas (TGF-βs) and their most specific signal transduction factors, Smads, are expressed in the endometrium during the window of implantation. Recent researches indicated that Smad dependent TGF-β signaling may play an important role in the process of embryo implantation. In this study, we measured the expression of TGF-β1, TGF-β receptor type I (TβRI), Smad3 and p-Smad3 in the endometrium of mice and observed their elevation on day 4, 5 and 6 of pseudopregnancy. Then we administrated a specific Smad3 inhibitor (Sis3) into the uterine cavity of mice on day 3 of pregnancy. The results showed a reduction in insulin-like growth factor-1 (IGFBP-1) expression and the decreased number of implanted embryo after the administration. In addition, Sis3 was found to reduce the IGFBP-1 secretion in decidualized endometrial stromal cells. Taken all together, our findings demonstrated that TGF-β/Smad3 signaling is involved in the process of embryo implantation.
Key words: transforming growth factor-betas (TGF-βs); Smad3; endometrial receptivity; decidualization; embryo implantation

Successful embryo implantation requires the interaction between the synchronously developed blastocyst and endometrium. However, the endometrium is only receptive to the blastocyst for a short period named the window of implantation, which spans from day 20 to 24 of the menstrual cycle[1, 2]. During this time, the local morphology and molecules, such as cytokines, growth factors and homeobox transcript factors, were accurately regulated, due to the regular fluctuation of ovarian steroid hormones[3, 4].

Transforming growth factor-betas (TGF-βs) are known to play multiple roles in many biological processes, including morphogenesis, embryonic development, immune regulation, inflammation and tumorigenesis[5, 6]. TGF-βs mainly binds to the type Ⅰ receptor (TβR1) and the type Ⅱ receptor (TβR2). It is known that TβR1 is activated by TβR2 after TGF-βs induce the phosphorylation of TβR2[7]. The knockout (KO) mouse model showed that absence of TβR1 led to vascular defects in placenta and finally caused fetal death at midgestation[8]. In female reproductive system, several studies have shown that the expression of all three TGF-β isoforms was elevated in the secretory phase and lowered in the proliferative phase of human endometrium[9, 10]. TGF-β receptors were also found to be highly and time-variably expressed in rat endometrial epithelial and stromal cells during the peri-implantation[11]. A recent study showed conditional KO of TβR1 in mouse endometrium resulted in abnormal implantation of blastocysts[12].

Smads are a group of transcriptional factors that specifically transduce extracellular signals from TGF-β superfamily ligands. Smad2 and Smad3, also called receptor-regulated Smad (R-Smads), are R-Smads of TGF-β subfamily and directly phosphorylated by TβR1[13]. Then the R-Smads form a complex with Smad4 (Co-Smad) and the complex finally binds to specific sites on the promoter of genes regulated by TGF-βs, with the aid of other co-factors like c-fos and c-jun[14]. Along with TGF-βs and their receptors, Smad2, Smad3 and Smad4 were also abundantly expressed in the endometrium of mice and rats during pre- and peri-implantation, as well as in the decidua tissue[15, 16]. It was also reported that Smad3 KO mice showed impaired decidualization[16]. Collectively, these findings indicate that the Smad-dependent TGF-β signaling transduction may play important roles in the process of embryo implantation. The main objective of this study was to clarify the expression pattern of

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the key proteins of TGF-β/Smad3 signaling during the implantation window of mice and investigate the role of this pathway in embryo implantation.

1 MATERIALS AND METHODS

1.1 Animals and Cell Lines

All animal experiments in this work were approved by the Animal Care and Use Committee of Tongji Medical College (China). ICR strain mice were purchased from the Center of Medical Experimental Animals of Hubei Province (Wuhan, China). The endometrial stromal cell line THESC (CRL-4003, ATCC) was kindly donated by the Department of Obstetrics and Gynecology, Justus-Liebig-University Giessen, Germany. All cells were cultured according to their guidelines in a cell culture incubator.

1.1.1 Animal Treatments and Tissue Preparation

Infertile male mice were established by performing vasectomy. Female mice (8–10 weeks) were mated naturally with infertile or fertile male mice under a standard condition of 12-h light and 12-h dark cycle. The presence of a vaginal plug next morning was considered as successful mating and that day was designated as Day 1 of pseudopregnancy or pregnancy. The uteri were obtained from unmated female mice or pseudopregnant mice on Day 4, Day 5 and Day 6 of pseudopregnancy, respectively (n=6). The endometrial tissues were then isolated from their uteri for quantitative real-time PCR. The pregnant mice were anesthetized with 2% pelltobarbitalum natricum (i.p. injection) on Day 3. The left side of each uterus was exposed and treated as described below: injecting 20 μL Sis3 (10 μmol/L, Calbiochem, Germany) through uterine horn (study group, n=12); injecting 20 μL vehicle through uterine horn (control group, containing the same amount of DMSO as in study group, n=12); puncturing the uterine horn with the injection syringe (sham group, n=12). After 4 h, three of these mice were sacrificed and their endometrial tissues were obtained and stored for Western blotting. The rest mice were sacrificed on Day 8 and their uteri were excised. Then the number of implanted embryos was counted.

1.2 Cell Culture and Treatments

For *in vitro* decidualization experiments, 5×10^5 endometrial stromal cells (THESC) were seeded into 6-well plates with DMEM/F12 medium (Hyclone, USA) and 10% FBS (Gibco, USA) and cultured in an incubator (37°C, 5% CO_2). On the next day, the cells were treated with or without 2.5 μmol/L Sis3. After 2 h, 0.5 mmol/L 8-Bromo-cAMP, 10 nmol/L 17β-estradiol-acetate and 1 μmol/L medroxyprogesterone acetate (MPA) (all from Sigma-Aldrich, USA) were added. After 3 days, the culture medium was collected and the stimuli were refreshed with fresh medium containing the same stimulants described above, respectively. The culture medium was collected again after 3 days. Cells without any treatment served as negative controls. The cell number was counted by using the Countstar cell counter (Ruiyu biotech, China). All the collected culture medium was centrifuged (5000× g) at 4°C for 10 min. Then the supernatants were aspirated and mixed with proteinase inhibitor cocktail (Sigma-Aldrich, USA) and stored at –20°C for further experiments.

1.3 ELISA

The quantity of insulin-like growth factor-1 (IGFBP-1) secretion was detected with human IGFBP-1 ELISA kit (Boster, China) according to the manufacturers' protocols respectively. The absorbance (A) values were obtained by using BioTek ELx808 (BioTek Instruments, USA). The cell number was used for standardization. All experiments were performed 3 times independently.

1.4 RNA Extraction and Quantitative Real-time PCR

Total RNA was extracted from the endometrial tissues by using TRIzol® reagent (Invitrogen, USA). Reverse transcription reaction was performed by using an RT kit (Takara, Japan) according to the manufacturer's protocol. The cDNA products were amplified by using lightcycler96® real-time quantitative PCR (Roche, Germany) with SYBR green Master Mix (Takara, Japan). The primer sequences are shown in table 1. For data analysis, the threshold was set based on the exponential phase of products and the $2^{-\Delta\Delta CT}$ method was performed. The expression level of TGF-β1, TβRI and Smad3 was normalized to that of GAPDH. All reactions were run in triplicate and all experiments were performed 3 times independently.

1.5 Western Blotting

The mouse endometrial tissues were scrapped and lysed in RIPA lysis buffer supplemented with protease inhibitor cocktail (Sigma-Aldrich, USA). The concentration of protein was determined by using the BCA protein assay kit (Pierce, USA). Afterwards, 40 μg of protein was loaded into each well of SDS polyacrylamide gel and was separated electrophoretically according to molecular mass. Then

Table 1 Primer sequences (5'– 3') used in quantitative real-time PCR

Genes	GenBank accession No.	Forward primer	Reverse primer
TGF-β1	NM_011577	ACCGCAACAACGCCATCTAT	GTAACGCCAGGAATTGTTGC
TβR1	NM_009370	CCAAACCACAGAGTAGGCAC	ACCAATAGAACAGCGTCGAG
Smad3	NM_016769	CATTCCATTCCCGAGAACAC	ATGCTGTGGTTCATCTGGTG
GAPDH	NM_008084	TCGGTGTGAACGGATTTGGC	GAATTTGCCGTGAGTGGAGT

the separated protein was transferred onto PVDF membranes (Millipore, USA) and blocked in TBST containing 5% nonfat milk at room temperature for one hour. Subsequently, the membranes were incubated with rabbit anti-Smad3, anti-phospho-Smad3, anti-IGFBP-1 or anti-GAPDH (1:1000 dilution, #9523, #9520, #31025, #5174, Cell Signaling Technology, USA) overnight at 4°C respectively, washed with TBST and incubated with HRP-conjugated anti-rabbit-IgG antibody. Immunoreactive bands were visualized by using enhanced chemiluminescence (Advansta, USA) according to the manufacturer's protocol. To evaluate the expression of target proteins, the protein bands were visualized using Genegnome XRQ (Syngene, UK) and the intensity of the bands was quantified with Gene Tools system (Syngene, UK).

1.6 Statistical Analysis

Graphpad Prism® (La Jolla, USA) was used for the statistical analysis. The numerical data were presented as mean±standard deviation (SD) and was compared by using unpaired *t*-test. *P* values less than 0.05 were considered statistically significant.

2 RESULTS

2.1 TGF-β1, TβRI and Smad3 mRNA Is Elevated in the Endometrium of Mice During Implantation Window

The expression of TGF-β1, TβRI and Smad3 mRNA was measured in the endometrium of unmated female mice or pseudo pregnant mice (n=6), respectively. The results showed that the mRNA expression levels of all the three factors were elevated on Day 4, Day 5 and Day 6 of pseudo pregnancy, when compared with those in the unmated female mice. However, the elevation on Day 6 was lowered, as compared with that on Day 4 or Day 5 (fig. 1).

2.2 Smad3 Pathway Is Activated in the Endometrium of Mice during Implantation Window

To determine whether the Smad3 pathway is activated during implantation window, the expression of Smad3 and p-Smad3 protein was measured in the endometrium of unmated female mice or pseudopregnant mice, respectively. Female mice were randomly divided into four groups (n=6). Then they were mated or not mated (control) with castrated male mice described in materials and methods. The results showed that the expression of both Smad3 and p-Smad3 was elevated in the endometrium of pseudopregnant mice on Day 4, Day 5 and Day 6, when compared with that in the endometrium of unmated female mice. The expression of both Smad3 and p-Smad3 reached the highest level on Day 4, and then declined gradually on Day 5 and Day 6 (fig. 2A, 2B). The p-Smad3/Smad3 ratio, which represents the phosphorylation level of Smad3 protein, was also increased on Day 4, Day 5 and Day 6 of pseudopregnancy (fig. 2C).

2.3 Administration with Sis3 Impedes Decidualization and Embryo Implantation *In Vivo*

The aforementioned results showed that the Smad-dependent TGF-β signaling pathway was activated during the implantation window of mice. To further evaluate whether Smad3 phosphorylation is involved in embryo implantation *in vivo*, Sis3, a specific inhibitor against Smad3 phosphorylation, was injected into the left uterine horn of mice. The expression of p-Smad3 was measured 4 h after Sis3 administration and a complete inhibition of Smad3 phosphorylation was observed. The IGFBP-1 expression was also down-regulated in Sis3 treated group (fig. 3A). The implanted embryos were counted on Day 8 of pregnancy. The number of implanted embryo in the Sis3 treated group was dramatically decreased, when compared with that in the DMSO treated group (2.22±1.30 *vs.* 5.44±1.24, P<0.001) and the sham group (2.22±1.30 *vs.* 7.11±1.36, P<0.001) (fig. 3B).

2.4 Smad3 Phosphorylation Is Essential for Decidualization *In Vitro*

To verify whether the Smad-dependent TGF-β signaling pathway is involved in decidualization *in vitro*, Sis3 was administrated into an *in vitro* decidual model using an endometrial stromal cell line (THESC).

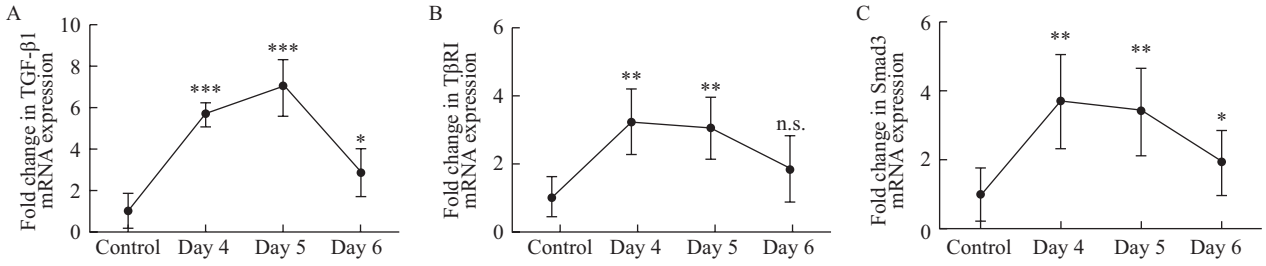

Fig. 1 qRT-PCR analysis of TGF-β1, TβRI and Smad3 mRNA expression in the endometrium of mice during implantation window The mRNA of all the three factors was expressed in the endometrium of unmated (control, n=6) and pseudopregnant mice (Day 4, Day 5 or Day 6, n=6). A: Compared with the control, the TGF-β1 mRNA level was elevated approximately 5.7-fold on Day 4, 7.0-fold on Day 5 and 2.9-fold on Day 6, respectively. B: The TβRI mRNA level was elevated approximately 3.2-fold on Day 4, 3.0-fold on Day 5 and 1.8-fold on Day 6, respectively. C: The Smad3 mRNA level was elevated approximately 3.7-fold on Day 4, 3.4-fold on Day 5 and 1.9-fold on Day 6, respectively ($^{***}P$<0.001, $^{**}P$<0.01, $^{*}P$<0.05 *vs.* control, n.s.: no significance).

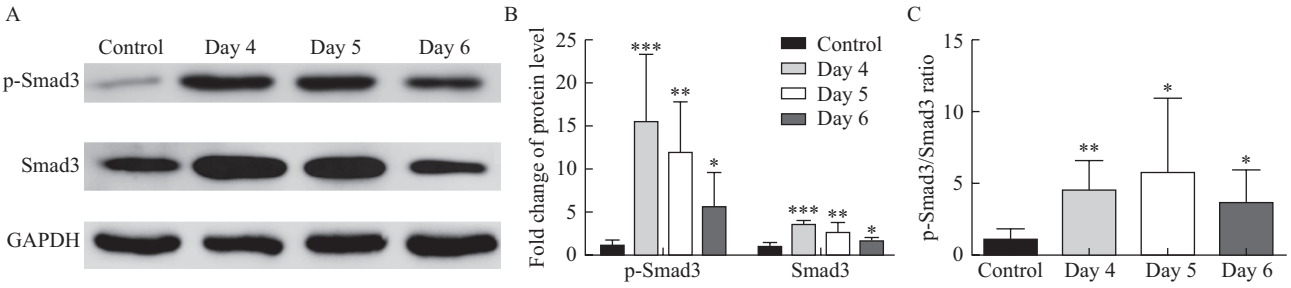

Fig. 2 Western blot analysis of Smad3 and p-Smad3 expression in the endometrium of mice during the implantation window
The protein expression of Smad3 and p-Smad3 was detected in the endometrium of unmated female mice (control, n=6) and pseudopregnant mice (Day 4, Day 5 or Day 6, n=6). A, B: The expression levels of Smad3 and p-Smad3 were both elevated on Day 4, Day 5 and Day 6 of pseudopregnancy, as compared with those in the control group. However, the increment declined on Day 5 and Day 6. C: The p-Smad3/Smad3 ratio was also enhanced on Day 4, Day 5 and Day 6 of pseudopregnancy ($^{***}P$<0.001, $^{**}P$<0.01, $^{*}P$<0.05).

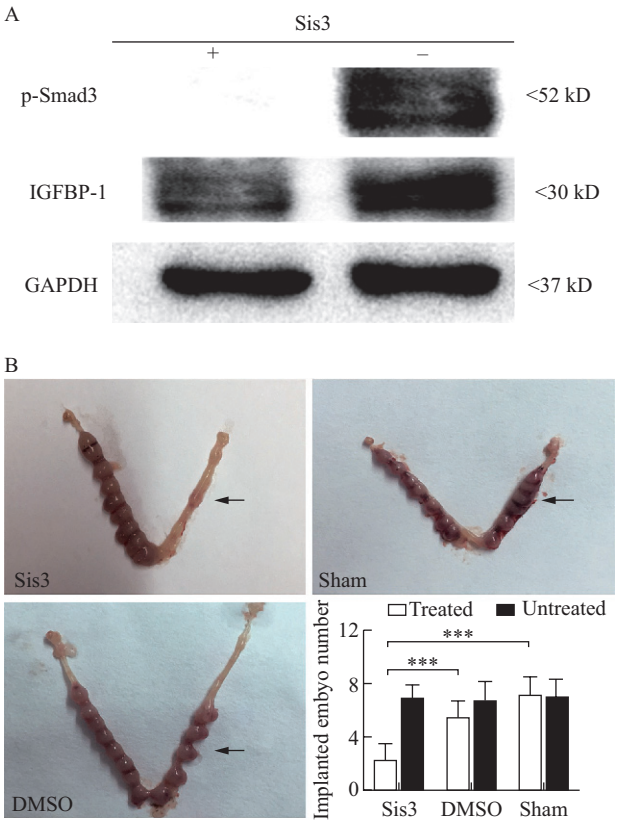

Fig. 3 Sis3 blocks Smad3 phosphorylation and impedes embryo implantation in mice.
A: The administration of Sis3 completely blocked the phosphorylation of Smad3 and decreased the expression of IGFBP-1 in the endometrium of mice in Sis3 treated group. B: Pregnant mice were divided into three groups randomly and treated as described in section Materials and Methods. The black arrow denotes the side of uterus that underwent the treatment. The other side (untreated side) was considered as self-control. The number of implanted embryo in Sis3 treated group (2.22±1.30, n=9) was much lower than that in either DMSO control group (5.44±1.24, n=9) or sham group (7.11±1.36, n=9). The number of implanted embryo in sham group was similar to its self-control, which proved that the uterine puncture did not affect embryo implantation ($^{***}P$<0.001).

The *in vitro* decidualization was established with the treatment of cAMP, E2 and MPA, and confirmed by detecting a dramatic increment of IGFBP-1 secretion on day 6 in cell culture. However, the IGFBP-1 secretion was remarkably lower in THESC cells with the presence of Sis3, compared with that without Sis3 treatment (fig. 4).

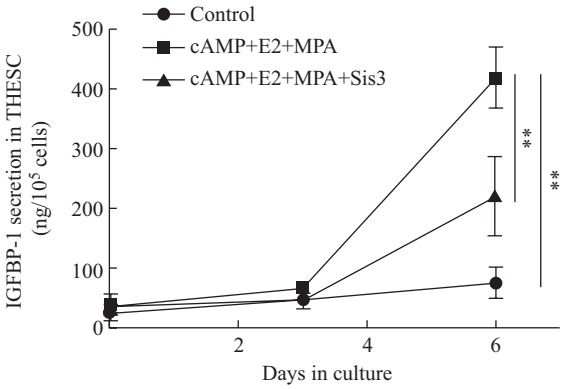

Fig. 4 Sis3 impedes decidualization of endometrial stromal cells *in vitro*.
The decidualization of endometrial stromal cells (THESC) was established by using E2, cAMP and MPA. IGFBP-1 was measured on Day 3 or Day 6 of the cell culture, respectively. A tremendous increase of IGFBP-1 secretion was observed on Day 6 of the cell culture, when compared with the control. However, the presence of Sis3 suppressed this increment approximately by half ($^{**}P$<0.01).

3 DISCUSSION

Embryo implantation is a crucial process of pregnancy, which depends on synchronous crosstalk between embryo and receptive endometrium. Multiple molecules and pathways are involved in this process and play pivotal roles in regulating endometrial receptivity. In this study, we investigated the expression and role

of Smad3 dependent TGF-β signaling pathway during implantation window in mouse endometrium. The results showed that activated Smad3 pathway was necessary for embryo implantation in mice.

Previous studies have shown that the expression of TGF-β isoforms reached their peak value during mid and late secretory phase in human endometrium[9, 10], which has shown their potential role in regulating endometrial receptivity. However, detailed alteration of these isoforms and their specific downstream transcription factors during the implantation window remains blurred. In this study, we measured the expression of TGF-β1, TβRI, Smad3 and p-Smad3 in the endometium during the implantation window of mice. Our results showed that their mRNA expression was elevated in this period. However, the elevation dropped on Day 6 when is considered to be the end of an implantation window in mice. These data showed that the expression of these genes might be correlated to the alteration of mouse endometrium during the implantation window. The protein level of Smad3 and its phosphorylation were both enhanced during the implantation window in mice. The enhancement was explicable since TGF-β1 was found to be activated from its latent complex during embryo implantation[17]. The activation of TGF-βs may lead to the phosphorylation of Smad3, which may play crucial roles in decidualization[16]. It had been reported that endometrial epithelial cell derived TGF-β1 enhanced stroma decidualization via Smad signaling[18]. It was also confirmed in this study that direct inhibition of Smad3 phosphorylation resulted in lower expression and secretion of decidualization marker IGFBP-1. These data demonstrated the important role of Smad3 pathway in maintaining endometrial stroma decidualization, which is an essential process for the subsequent trophoblast invasion[19].

To verify whether the activation of Smad3 pathway influences embryo implantation in mice, we inhibited its phosphorylation in the uterine of mice before the blastocysts entered the uterine cavity. Adequate controls were set to diminish the influence from operation and drug solvent. The results showed a decreased number of implanted embryos after Sis3 treatment. These data demonstrated that the activation of Smad3 pathway was crucial for the endometrium to accept embryos. Smad3 is one of the most important signal transductor in TGF-β signaling which influences the transcription of TGF-β responsive genes[7, 13]. Some of them like integrins and extracellular matrix were believed to be critical for endometrial receptivity[20–22]. Besides, matrix metalloproteinases (MMPs) and their natural inhibitors tissue inhibitor of metalloproteinases (TIMPs) contribute to the endometrial extracellular matrix remodeling and trophoblast invasion during peri-implantation period[19, 23]. It was reported that TGF-β1 remarkably increased the mRNA level and activity of MMP2 and MMP11 in human endometrial stomal cells[24]. Thus, blocking Smad3 phosphorylation may interrupt the regulation of TGF-βs on these genes and finally affect embryo implantation.

In conclusion, this study revealed the expression features of key factors in TGF-β/Smad3 pathway during the implantation window in the endometrium of mice and found that TGF-β/Smad3 pathway was enhanced and activated during this period, suggesting the important role of TGF-β/Smad3 pathway during embryo implantation. The results of animal experiments validated that the activation of this pathway in endometrium is critical for embryo implantation in mice. However, further studies are required to discover more downstream mechanisms.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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