

Original Article

Smad signaling coincides with epithelial-mesenchymal transition in a rat model of intrauterine adhesion

Luo-Pei Guo¹, Li-Mei Chen¹, Fang Chen¹, Ning-Hong Jiang¹, Long Sui^{1,2}

¹Obstetrics and Gynecology Hospital of Fudan University, Shanghai 200011, China; ²Shanghai Key Laboratory of Female Reproductive Endocrine Related Diseases, Shanghai 200011, China

Received January 26, 2019; Accepted August 4, 2019; Epub August 15, 2019; Published August 30, 2019

Abstract: Purpose: Intrauterine adhesion (IUA) is a fibrotic disease mainly caused by tissue injury, yet the mechanism is poorly understood. The aim of this study was to investigate the roles of TGF- β 1/BMP7/Smad signaling coincident with epithelial-mesenchymal transition (EMT) in IUA. Methods: Twenty-four female SD rats were divided into IUA and sham groups. For each animal, a mechanical injury or sham operation was performed on the left uterus (IUA-L, Sham-L), and the right uterus (IUA-R, Sham-R) was used as the control. Animals were sacrificed in batches on days 7 and 28. The endometrial morphology, number of endometrial glands, microvascular density (MVD), area of endometrial fibrosis and immunohistochemistry (IHC) analysis of biomarkers of EMT, as well as levels of TGF- β 1, phosphorylated Smad3 (pSmad3), BMP7, phosphorylated Smad1/5 (pSmad1/5) and estrogen receptor (ER) were evaluated. Besides, the correlation between these IHC markers was also analyzed. RT-PCR and western blot were used to test relevant genes. Results: Compared with other groups, the IUA-L group showed a significant decrease in the number of glands and MVD. And it also showed a significant increase in the stromal fibrosis rate and α -SMA level. Moreover, in the IUA-L group, TGF- β 1 and pSmad3 levels were consistently high, and levels of BMP7, pSmad1/5 and ER were low. EMT markers E-cadherin was decreased, while N-cadherin was increased. Sham and control groups showed no significant difference in these markers. In addition, E-cadherin with α -SMA, fibrosis rate with BMP7, TGF- β 1 with pSmad3 and BMP7 with pSmad1/5 showed correlation in IUA-L group, which had statistical significance. The mRNA expression of TGF- β 1, α -SMA and *ccn2* in 7 d IUA-L was higher than 7 d IUA-R while BMP7 was lower, which had significant difference. The protein expression of BMP7 in 7 d IUA-L was lower than 7 d IUA-R, which had significant difference. Conclusions: These results suggest a potential role of Smad signaling together with EMT in endometrial fibrosis development.

Keywords: IUA, TGF- β 1, BMP7, EMT, fibrosis, Smad

Introduction

Intrauterine adhesion (IUA) is characterized by the presence of menstrual irregularities, infertility and recurrent pregnancy loss and is drawing increased attention as a serious complication of uterine surgery. The prevalence of IUA after termination of pregnancy (TOP) varies from 8.1% to 21.2% according to different studies [1-3]. In addition, the recurrence rate of IUA can be as high as 62.5% [4]. However, the current standard treatment is not satisfactory, especially for severe IUA, yet little is known about the mechanism underlying IUA.

Most surgeons take IUA as a fibrotic disease for granted since endometrial cells lose sensitivity

to hormones and become fibrosed histologically [5]. As a matter of fact, epithelial-mesenchymal transition (EMT) is acknowledged to play a vital role in organ injury and fibrogenesis due to trauma [6]. Not surprisingly, researchers easily connected EMT to the development of endometrial fibrosis. Based on a report indicating that mesenchymal protein vimentin increased while epithelial marker cytokeratin decreased in IUA mouse models [7]. In addition, transforming growth factor β 1 (TGF- β 1), an archetypical promoter of fibrosis, was observed being significantly elevated in human IUA samples [8]. Moreover, Salma et al provided specific proof suggesting that TGF- β 1 may influence endometrial fibrosis via Smad3 both in IUA patients and rabbit models but did not explain the connection

Smad signaling coincides with EMT in IUA rat model

with EMT [9]. Besides, these researches never illustrate the location of EMT markers in IUA endometrium. Therefore, our study explored the EMT markers' location of fibrosed and normal endometrium to prove whether EMT function in endometrial fibrosis for the first time.

Bone morphogenetic protein 7 (BMP7), a member of the TGF- β superfamily, has been admitted by FDA for plastic surgery use [10]. Members of this subfamily were first identified in bone and thought to induce osteogenesis; however, increasing evidence has subsequently indicated their fundamental roles in organ homeostasis [11]. These findings enlightened us that BMP7 may also inhibit the fibrous progression of IUA.

In fact, these studies inspired our group to investigate the relationship between TGF- β 1/BMP7 and EMT. We hypothesized that TGF- β 1 counterbalances BMP7 through Smad signaling in the development of endometrial fibrosis coinciding with EMT.

Materials and methods

Animals and experimental protocol

This study was approved by the institutional ethics review board of Shanghai OB/GYN Hospital. Six- to eight-week-old sexually mature female rats were purchased from Silaike Corporation (Shanghai, China). Each rat was provided with chow and fresh water in the same controlled conditions (20°C, 12 h:12 h light/dark cycle with lights on at 6:00 am).

All the rats were anesthetized by pentobarbital injection and placed in the supine position, and the abdominal area was shaved and disinfected with povidone-iodine. The uterine horns were identified after a vertical incision was made in the midline of the lower abdomen. In the IUA group, the left uterus was operated on through a 2.5 cm longitudinal incision (**Figure 1A-C**). Here, we chose a mechanical injury method that used a pair of scissors to destroy the endometrium following a method described in our previous work [12]. In the sham group, rats received only an incision wound without any treatment to the endometrium. After the intervention, the uteri were sutured and gently moved back to their pelvic position. Six rats from each group were sacrificed on days 7 and 28.

Hematoxylin & eosin (HE) staining

Uteri were fixed in 4% paraformaldehyde for 24 h and embedded in paraffin. The slides were first deparaffinized and rehydrated, and then they were stained with hematoxylin and eosin. We visualized the whole image of the pathological uterus and counted the gland quantity to predict IUA.

Masson staining

Masson staining was used for the detection of endometrial fibrosis. The deparaffinized and rehydrated slides were incubated with Masson staining mixture for 5 minutes and then stained with phosphomolybdic acid-aniline blue solution for 6.5 minutes. The areas of the collagen fibers stained in blue relative to the total view were calculated by Image Pro-Plus 6.0.

Immunohistochemistry

After deparaffinization, dehydration and antigen retrieval, the slides were immersed in 3% hydrogen peroxide to block endogenous peroxidase activity and then blocked in goat serum for 1 hour. The slides were then incubated overnight at 4°C with the primary antibodies against CD31 (Abcam, 1:200), α -smooth muscle actin (α -SMA, Abcam, 1:200), E-cadherin (Santa Cruz Biotechnology, Inc., 1:100), N-cadherin (Servicebio, Inc., 1:200), TGF- β 1 (Abcam, 1:200), BMP7 (Abcam, 1:200), pSmad3 (Bioss, Inc., 1:300), pSmad1/5 (Bioss, Inc., 1:200) and estrogen receptor (Abcam, 1:200). Biotinylated goat anti-mouse or goat anti-rabbit secondary antibody (Jiehao) was applied for 60 min at room temperature. The slides were later stained with 3,3-diaminobenzidine (DAB) at room temperature, lightly counterstained with hematoxylin, dehydrated and covered with glass cover slips. Quantification of immunoreactivity was performed by Image Pro-Plus 6.0, and 3-5 fields were randomly selected from each slide to determine the mean optical density (MOD).

Immunofluorescence

Immunofluorescence staining was carried out for α -SMA (myofibroblast marker) and vimentin (mesenchymal marker). After antigen retrieval and blockade of nonspecific binding sites, the slides were incubated overnight at 4°C with primary antibodies. For detection, the sections

Smad signaling coincides with EMT in IUA rat model

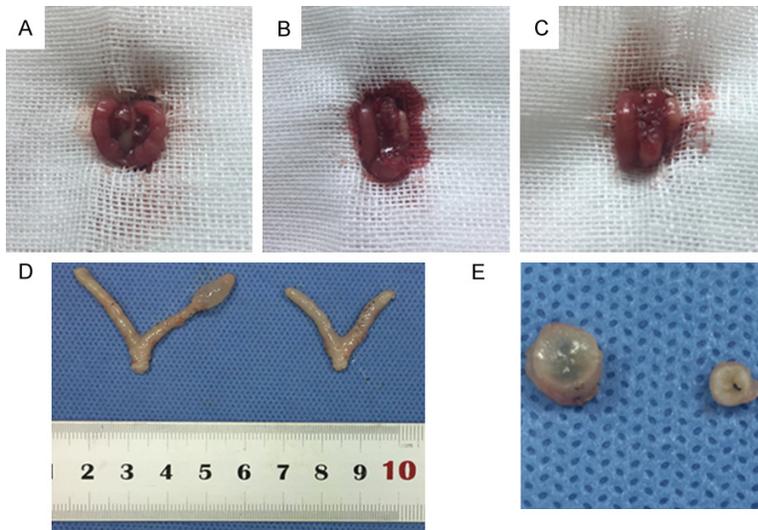


Figure 1. Mechanical surgery to establish an IUA rat model. A. Normal rat uterine appearance. The uterine had two horns and the color appeared red. B. After disinfection, tweezers were used to pick Y-shaped uterus, the endometrial surface was damaged by mechanical injury. Using scissors to cut endometrium layer carefully, kept the depth to superficial muscle layer. Sterile gauzes were needed to make the surgical area clean. C. Suture after the operation. The injured uterus looked distorted and unshapely. D. The profile of rat uteri on day 7, hydrops was found in IUA-L uterus group as the Sham-L group showed no difference with IUA-R and Sham-R group. E. IUA-L uterus was blocking and IUA-R uterus enjoyed a normal uterine cavity.

were exposed to Alexa 488 goat anti-rabbit IgG secondary antibody (1:1000 dilution, Abcam) for 60 min. Counterstaining of nuclei with 4',6-diamidino-2-phenylindole (DAPI) was also performed.

Confocal microscopy

Fluorescent slides were examined by a confocal laser scanning microscope (Leica TCS SP5 confocal microscope, Solms, Germany). Images were recorded with objective lenses (100 \times objective), and then exported as a TIFF-format digital file.

Quantitative real-time RT-PCR

Grind the excised uterine tissues using Trizol reagent (Invitrogen), following the manufacturer's instructions. The purity and concentration of RNA were identified then reverse transcribed to cDNA. Quantitative real-time PCR using 384-well optical plates was performed in a SYBR green (Takara, Japan) format with 10 μ L template. Each group included 6 samples, and each sample was done in triplicate. We used $2^{-\Delta\Delta CT}$ method to analyze relative gene expression.

Western blot analysis

Total proteins of uterine tissues using RIPA (Beyotime) with PMSF (Beyotime) were extracted. The concentration of protein was quantified by BCA kit (Beyotime). 30 μ g proteins were added to SDS-PAGE for separation, subsequently blotted on to PVDF membrane and incubated with primary antibody BMP7 (Abcam, 1:1000). The membrane was washed and treated with second antibody. Finally, the membrane was scanned with a Darkroom Eliminator.

Statistical analysis

All measurement data are shown as the mean \pm sd. T-test and One-way analysis of variance was used to analyze the data from different groups, followed by a Bonferroni test for post hoc comparisons.

Spearman's rank correlation coefficient was used when evaluating correlations between two variables. The data were analyzed by SPSS 20.0. $P < 0.05$ was defined as indicative of statistical significance.

Results

Endometrial morphology of the rat model

The sham and control endometria were composed of four to six polypoid protrusions with the surfaces covered by columnar epithelial cells. Most endometrial glands were located in the functional and basal layers. Seven days after the IUA operation, we observed hydrops of the uteri in the flesh (**Figure 1D** and **1E**). According to the HE staining, the uterine cavities were totally blocked, almost all the surface epithelium had disappeared, and the glands were hard to detect both in the functional and basal endometrial layers. The stroma became edematous, and extracellular matrix (ECM) collagen depositions increased. Moreover, the capillaries were broken and formed congestion, and severe hemorrhaging and neutrophil cells infiltrated in the stroma (**Figure 2A** and **2B**). At

Smad signaling coincides with EMT in IUA rat model

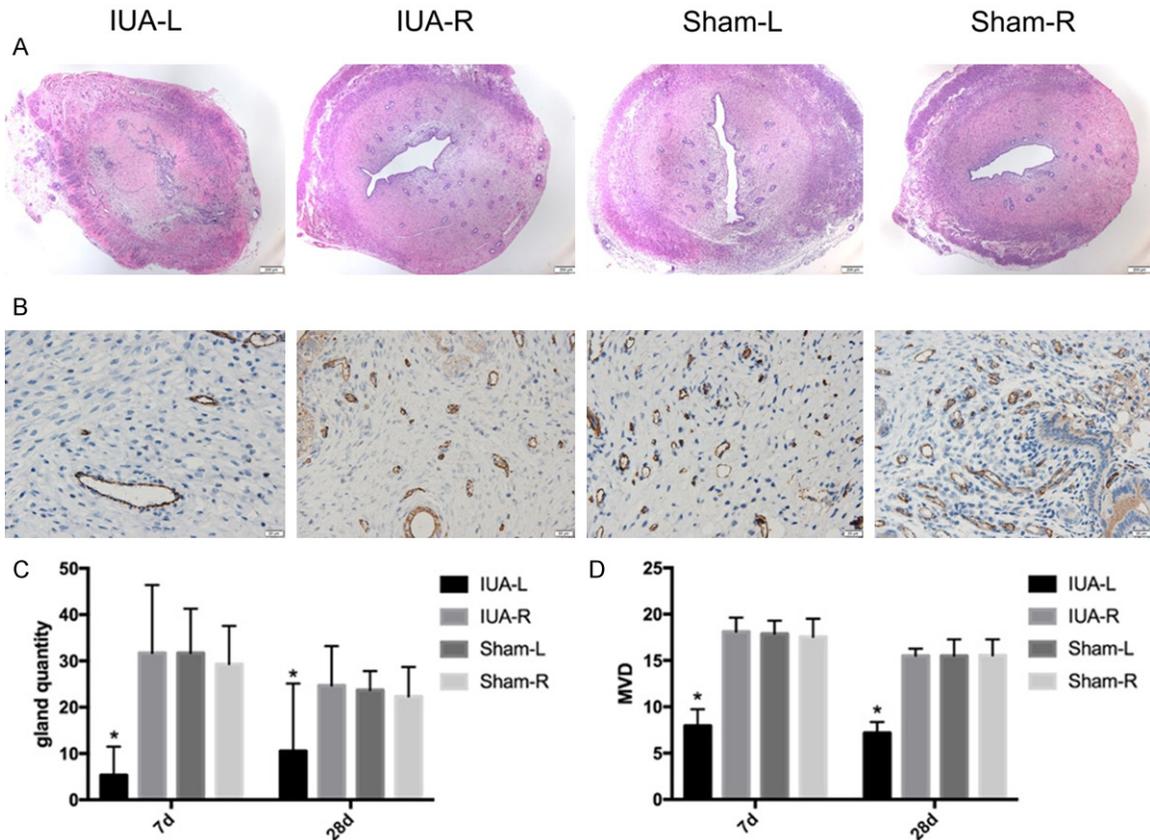


Figure 2. The micro view of an IUA rat model. A. HE staining of rat uteri after 7 days; the IUA-L uterine cavity totally disappeared and gland quantity decreased sharply. Neutrophil cells and red blood cells also filled in IUA-L endometrium. Scale bar = 200 μ m. B. Immunohistochemical staining of the endometrium for CD31. Light yellow circle symbolized as active capillary, MVD was counted by CD31. Scale bar = 20 μ m. C. Gland quantity decreased significantly in the 7 d and 28 d IUA-L group compared with that of other groups, $P < 0.05$. Other groups showed no differences. D. MVD decreased significantly in the 7 d and 28 d IUA-L group compared with that of other groups, $P < 0.01$. Other groups showed no differences.

28 days after surgery, the IUA-L uterine horns recovered from acute inflammation, and a minority of the endometria had re-epithelized; however, hydrops has become more severe, and ruptured vessels and ECM still existed. Moreover, there appeared to be fibrinoid degeneration in the stroma. In addition, the numbers of glands ($P < 0.05$) and MVD (CD31) ($P < 0.01$) on days 7 and 28 were significantly reduced in the IUA-L group compared with those in the IUA-R and sham group (**Figure 2C** and **2D**).

Endometrial fibrosis in the IUA rat model

As fibrosis is of considerable importance in the development of IUA, we performed Masson staining and a-SMA immunoreactivity to assess the extent of endometrial fibrosis extent, we further detected fibrosis genes expression as

well. The endometrial cells, blood vessels and muscle were dark red, but the endometrial stromal fibers appeared blue after Masson staining (**Figure 3B**). In addition to the myometrial cells, a-SMA was also seen in the fibrous stroma. Here, we provided a confocal image of a-SMA for a better view but still quantified a-SMA by IHC (**Figure 3A**). All these methods indicated that IUA rat uteri contained increased levels of collagen fibers and a-SMA while the staining was negative in the control and sham uteri. We calculated the proportion of fibrotic tissues based on Masson staining and a-SMA level by IHC. The ratio of endometrial fibrosis was highest in the IUA operated side, and the other groups showed no significant differences ($P < 0.01$, **Figure 3C**). Similar results were found for the a-SMA level ($P < 0.01$, **Figure 3C**). Moreover, the fibrous gene level of *ccn2* (also known as

Smad signaling coincides with EMT in IUA rat model

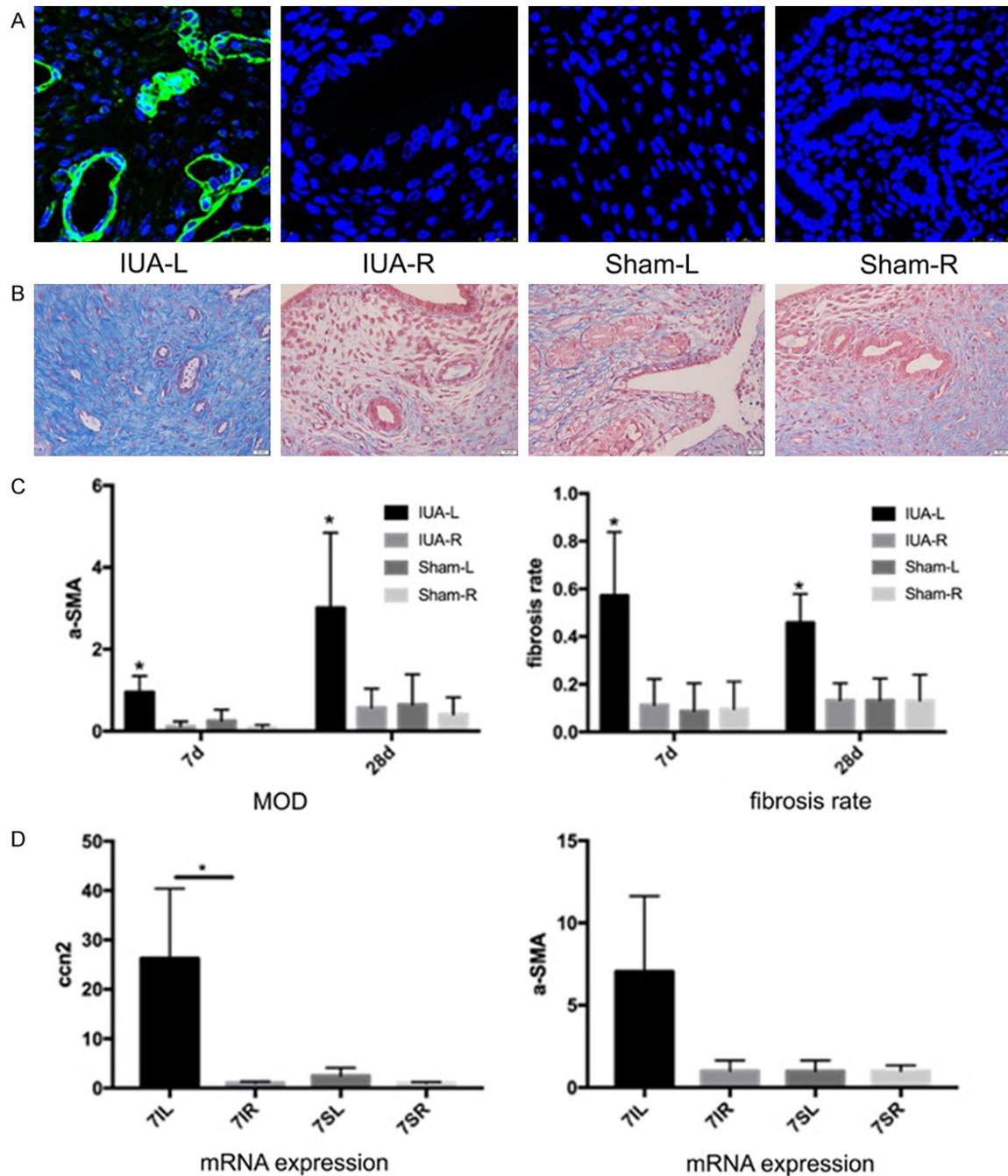


Figure 3. The IUA rat model became fibrotic. A. Typical a-SMA green staining of IUA-L stroma on day 7, IUA-R, Sham-L and Sham-R endometrium did not show positive immunofluorescence staining. a-SMA was not normally expressed in endometrial layer. But in IUA-L group, a-SMA was seen in the fibrous stroma. Here, we provided a confocal image of a-SMA for a better view but still quantified a-SMA by IHC. Scale bar = 25 μ m. B. Masson staining of the endometrium 7 days after surgery in the IUA and sham groups. The extent of blue color in endometrial layer meant deeper fibrosis rate, as we can see, IUA-L group showed much deeper blue than IUA-R, Sham-L and Sham-R group. Besides, less glands and epithelial cells were found in IUA-L group. Scale bar = 20 μ m. C. IHC staining of a-SMA in the IUA-L group was highest among the groups, $P < 0.01$. According to Masson staining, the average fibrosis rate was $57.17\% \pm 0.2658$ and $45.81\% \pm 0.1209$ in IUA-L on day 7 and day 28, $P < 0.01$, respectively. D. The mRNA expression of *ccn2* and a-SMA were relevant fibrosis markers. The mRNA level of *ccn2* in 7 d IUA-L group was 25 folds meaningfully higher than 7 d IUA-R group. Even though the mRNA expression of a-SMA showed no difference between 7 d IUA-L and IUA-R group, we could still observe the potential higher expression. As for Sham-L and Sham-R group, mRNA expression of *ccn2* and a-SMA both showed no significant difference.

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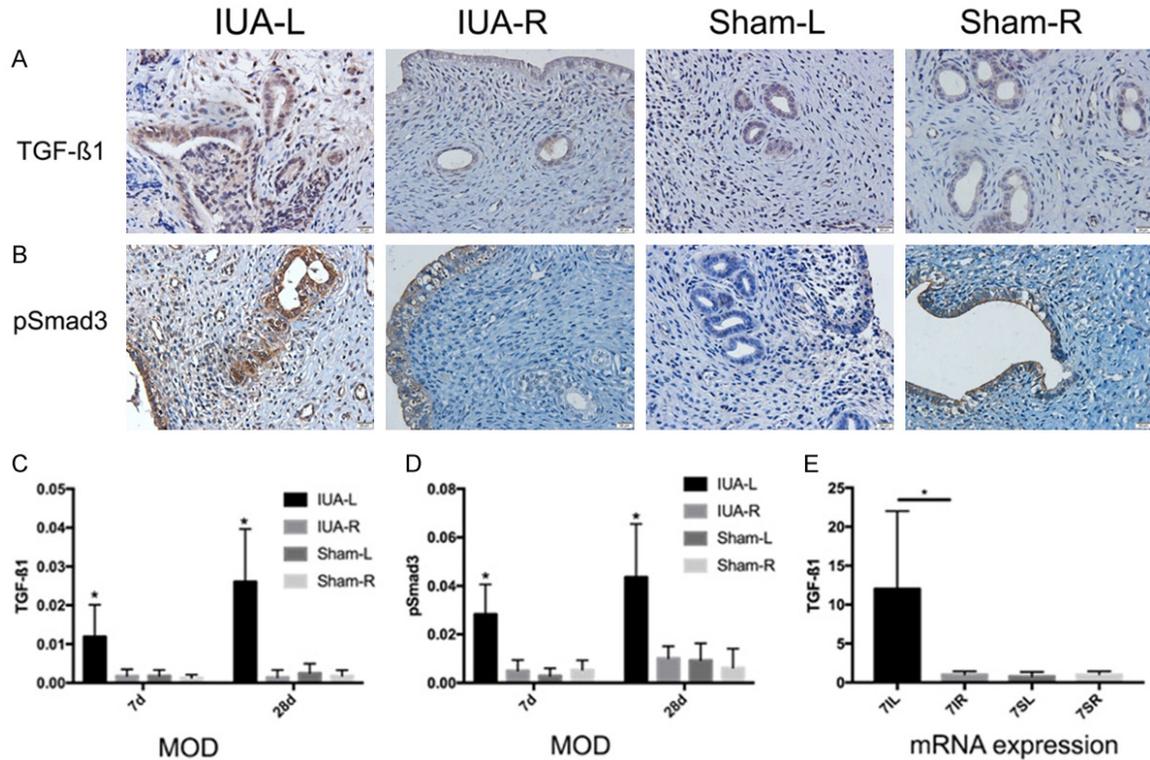


Figure 4. Representative immunostaining of TGF- β 1 and pSmad3 in rat model on day 7. A. TGF- β 1 staining was increased progressively in the epithelial cell cytoplasm in the IUA-L uterus but was negative in IUA-R and sham uteri. Scale bar = 20 μ m. B. pSmad3 staining was positive in the nuclei of epithelial cells and a small number of stromal cells in the IUA-L group but negative in the normal endometrium too. The location and expression of TGF- β 1 and pSmad3 were identical in IUA-L group. Scale bar = 20 μ m. C. The *P* value of the differences in TGF- β 1 was $P < 0.01$ both on day 7 and was $P < 0.05$ on day 28. D. The *P* values of the differences in pSmad3 on day 7 and day 28 were $P < 0.01$. Scale bar = 20 μ m. E. The gene level of TGF- β 1 was more 10 folds higher in 7dL compared with 7dR group ($P = 0.0390$). This was consistent with immunostaining of TGF- β 1.

CTGF, connective tissue growth factor) and α -SMA were highly expressed in day 7 IUA-L (7dL) group (Figure 3D). The expression of *ccn2* was significantly increased in 7dL ($P = 0.0159$) while α -SMA had a tendency of high expression in 7dL ($P = 0.1775$).

Activation of Smad signaling in the rat model of IUA

TGF- β 1 immunoreactivity was increased progressively mainly in the epithelial cell cytoplasm in the IUA-L uterus but was negative in IUA-R and sham uteri (Figure 4A). Besides, the gene level of TGF- β 1 was also highly expressed in 7dL compared with 7dR groups, which had a significant difference ($P = 0.0390$, Figure 4E). As for pSmad3 staining, it was positive in the nuclei of epithelial cells and a small number of stromal cells in the IUA-L endometrium but neg-

ative in the normal endometrium too (Figure 4B). The expression of these two markers were significantly increased as IUA developed in comparison with those of controls ($P < 0.05$, Figure 4C and 4D). Furthermore, the staining intensity for BMP7 was decreased significantly in the stromal cell cytoplasm in the IUA-L while it was still strong in IUA-R and sham endometrium ($P < 0.01$, Figure 5A). Moreover, pSmad1/5 was also significantly decreased both in the nuclei of epithelial and stromal cells in the IUA-L endometrium when compared with normal endometrium ($P < 0.01$, Figure 5B). And the gene and protein expression of BMP7 were significantly decreased in 7dL compared with 7dR ($P = 0.0286$, $P = 0.0214$, Figure 6). Likewise, pSmad3 staining showed almost identical results with TGF- β 1, and pSmad1/5 was closely connected with BMP7, which indicates an activation of the TGF- β 1/pSmad3 and BMP7/

Smad signaling coincides with EMT in IUA rat model

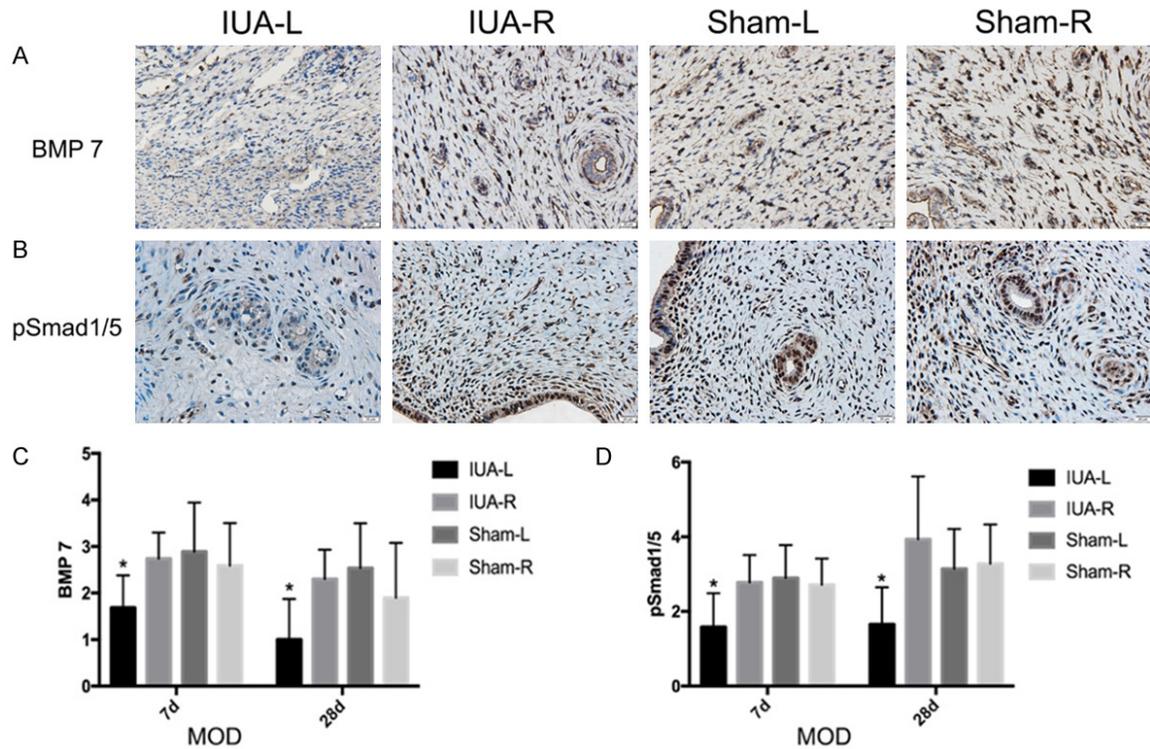


Figure 5. Representative immunostaining of BMP7 and pSmad1/5 in rat model on day 7. A. BMP7 was fully expressed in the stromal cell cytoplasm. The immunostaining intensity of BMP7 in IUA-L decreased, it was still strong in IUA-R and sham endometrium. B. pSmad1/5 was also located in the nuclei of epithelial and stromal cells. And it showed less positive in IUA-L endometrium when compared with IUA-R, Sham-L and Sham-R. BMP7 and pSmad1/5 were similarly decreased in the IUA-L group but maintained high expression in the other groups. C, D. The MOD of BMP7 and pSmad1/5 were lower than other groups. The *P* value of the differences in BMP7 and pSmad1/5 was $P < 0.05$ on day 7 and day 28. Scale bar = 20 μm .

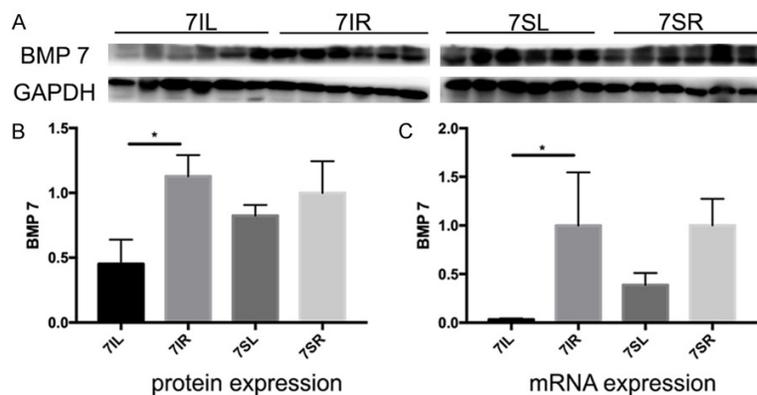


Figure 6. The mRNA and protein expression of BMP7. A. 6 samples were collected from each group. Western blot membrane from left to right represented 7IL, 7IR, 7SL and 7SR. The first row was BMP7 protein marker, and the second row was their GAPDH. B. T-test analysis proved BMP7 protein expression was lower in 7IL compared with 7IR ($P = 0.0286$). As 7SL showed no significant difference with 7SR. C. The mRNA expression of BMP7 was significantly decreased in 7IL compared with 7IR ($P = 0.0214$). While 7SL and 7SR had no statistical difference.

EMT-associated proteins during the IUA development and ER expression

Membranes of epithelial cells of normal endometrium stained positive for E-cadherin (Figure 7A), while N-cadherin was rarely present in normal endometrium; however, in our study, the membrane of IUA model uterine epithelial cells stained positive for N-cadherin (Figure 7B). IHC revealed a reduction in the epithelial marker protein E-cadherin consistent with an increase in the mesenchymal marker protein N-cadherin in the IUA-L endometrium compared with the IUA-R and sham ones ($P < 0.01$, Figure 7C, 7D). Vimentin,

pSmad1/5 signaling pathway developing in endometrial fibrosis.

which was localized mostly to the cytoplasm of stromal cells in both normal and model endo-

Smad signaling coincides with EMT in IUA rat model

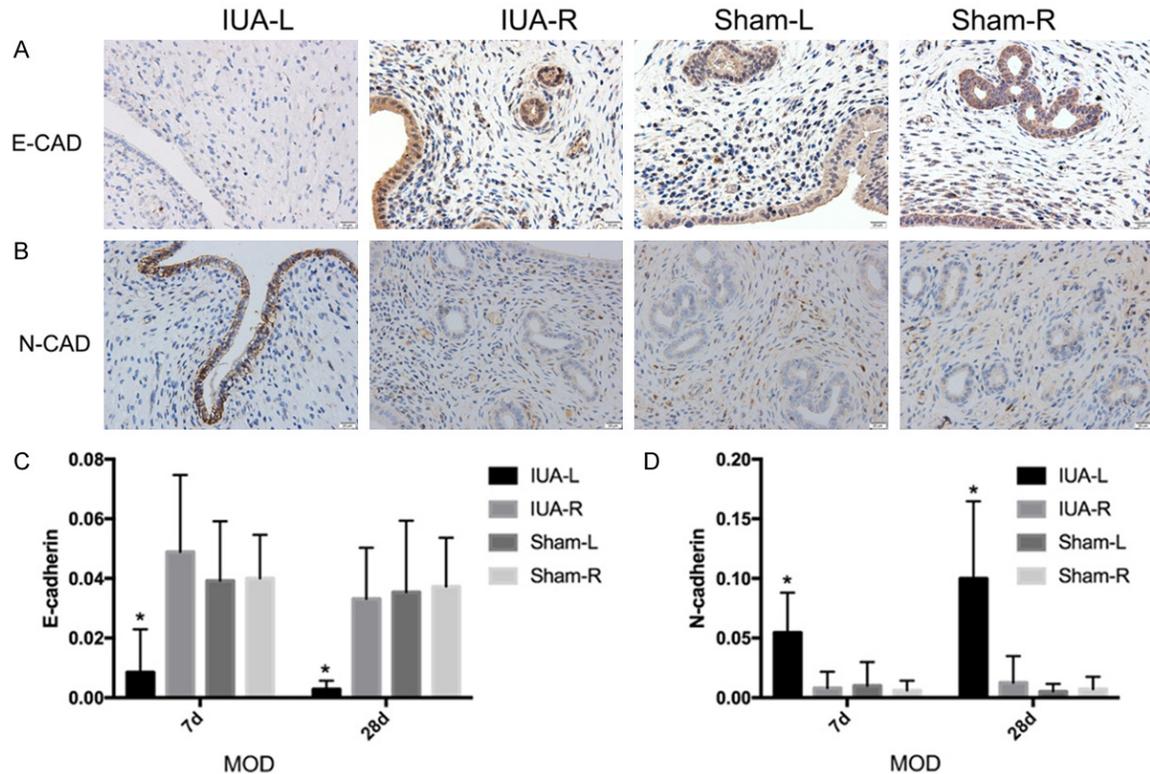


Figure 7. EMT markers changed in the IUA-L group. A. Usually, E-cadherin affluently displayed in normal epithelial cells. After the mechanical injury, it was hard to detect E-cadherin staining in IUA-L. Scale bar = 20 μ m. B. N-cadherin was an epithelial marker but rarely expressed in epithelial cells. This study found strong N-cadherin staining in IUA-L epithelial cells but light staining in normal epithelial cells. Scale bar = 20 μ m. C. The MOD level of E-cadherin after day 7 and day 28 declined significantly in the IUA-L group, $P < 0.01$. D. N-cadherin level after day 7 and day 28 increased significantly in the IUA-L group compared with that of other groups, $P < 0.01$.

metrium, was increasingly seen in the cytoplasm of epithelial cells in the model uteri directly from the fluorescent images (**Figure 8A**). Altogether, these results suggest that the EMT phenomenon may influence endometrial fibrosis. In addition, we also tested ER level of endometrium. ER, closely correlated with sexual hormone, was usually located in the nuclear of stromal cells. After endometrium injury, ER expression decreased obviously in IUA-L when compared with IUA-R and sham groups ($P < 0.001$, **Figure 8B** and **8C**).

Relationship between EMT and Smad signaling

We found that EMT marker E-cadherin in IUA-L group correlated with fibrosis marker α -SMA ($r = 0.533$, $P < 0.000$). Fibrosis rate (Masson staining) was correlated with BMP7 ($r = 0.333$, $P = 0.048$) and TGF- β 1 was correlated with pSmad3 staining levels ($r = 0.424$, $P = 0.001$)

in IUA-L group. BMP7 was correlated with pSmad1/5 staining levels in IUA-L group ($r = -0.398$, $P = 0.004$) and Sham-L group ($r = 0.561$, $P < 0.000$). All these markers showed no correlation among other groups (**Table 1**).

Discussion

This study investigated the fibrotic mechanism of IUA on Smad signaling coincident with EMT. IUA is a clinical complication mostly caused by dilation and curettage. As such, IUA can be viewed as wounds that undergo tissue injury and repair. Indeed, menstruation can be regarded as a physiological process, but IUA is a more serious inflammatory process and causes fibrosis [13]. Since TGF- β 1 was closely connected with inflammation, more studies have reported that TGF- β 1 was increased both in IUA animal models and human endometrial samples. And it may enhance IUA fibrosis through Smad3 signaling [8, 14-17]. Our study

Smad signaling coincides with EMT in IUA rat model

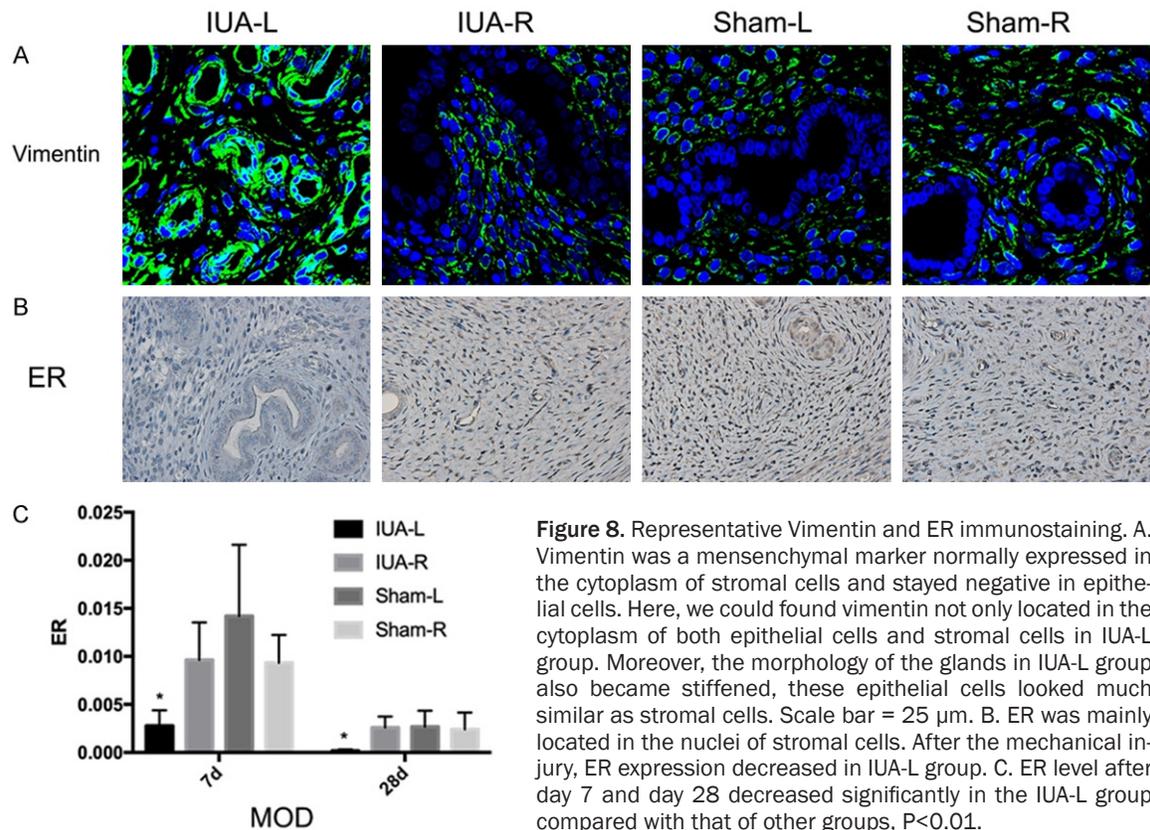


Figure 8. Representative Vimentin and ER immunostaining. A. Vimentin was a mesenchymal marker normally expressed in the cytoplasm of stromal cells and stayed negative in epithelial cells. Here, we could find vimentin not only located in the cytoplasm of both epithelial cells and stromal cells in IUA-L group. Moreover, the morphology of the glands in IUA-L group also became stiffened, these epithelial cells looked much similar as stromal cells. Scale bar = 25 μ m. B. ER was mainly located in the nuclei of stromal cells. After the mechanical injury, ER expression decreased in IUA-L group. C. ER level after day 7 and day 28 decreased significantly in the IUA-L group compared with that of other groups, $P < 0.01$.

Table 1. Statistical significance in immunoreactivity correlation for rat model

IHC marker	IUA-L	IUA-R	Sham-L	Sham-R
Spearman's rank correlation ($r =$)				
<i>(P-value)</i>				
E-cad/a-SMA	0.533***	0.183	0.186	0.240
	<0.000	0.286	0.277	0.165
Masson/BMP7	0.333*	-0.124	-0.280	-0.063
	0.048	0.473	0.098	0.714
TGF- β 1/pSmad3	0.424**	-0.275	0.202	0.175
	0.001	0.061	0.150	0.190
BMP7/pSmad1/5	-0.398**	-0.107	0.561***	-0.216
	0.004	0.456	<0.000	0.137

The first column illustrated protein name. The first row of protein was r coefficient, the second row of protein was P -value. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; P -value were derived by Spearman's rank correlation coefficient.

proved that TGF- β 1 closely connected with pSmad3 on endometrial fibrosis as the most classical signaling. Here we used a mechanical injury rat model, which may better imitate IUA etiology, though other researches have used the chemical constituent like lipopolysaccharide (LPS), or an electrothermal injury method [18, 19].

The TGF- β family includes over 30 members, including three TGF- β s, one Nodal protein, eleven growth and differentiation factors (GDF), four activins and ten BMPs [20]. To our surprise, there are very few family members, including BMPs, that have been studied in the endometrium, yet the TGF- β superfamily members are such common and essential differentiative and proliferative factors among organisms [21].

To our knowledge, BMP2 is critical for decidualization and embryo implantation [22]. In addition, BMP4 is closely connected with embryonic development and organ formation. In addition, a recent report has shown that BMP4 may be vital in controlling sex hormones and protecting against hyperandrogenism [23]. As for BMP7, which has been indicated in the inhibition of decidualization and proliferation of endometrial stromal cells and may be decreased by progesterone [24]. In addition, abnormal uterine bleeding is also associated with increased BMP7 in human endometrium [25]. As mentioned above, BMP7 possesses anti-inflammatory and anti-

brotic properties [26]. In our study, BMP7 exactly decreased in IUA-L group both in mRNA and protein level. What's more, BMP7 was connected with fibrosis rate, and it was highly expressed in normal rat endometrium too, especially in stromal cells. The level of pSmad1/5, consistent with that of BMP7, was decreased in the IUA endometrium. Furthermore, BMP7 negatively correlated with pSmad1/5 in IUA-L group. But they were positively correlated in Sham-L group. On the one hand, it may suggest that BMP7 Smad signaling failed to perform its antifibrotic role in IUA-L group but the two variables were still closely connected. On the other hand, it indicated BMP7 Smad signaling well performed its antifibrotic role in Sham-L group. All these phenomena suggested that a BMP7/Smad1/5 signaling may exist in endometrial fibrosis.

EMT is the functional loss of E-cadherin in epithelial cells and the concomitant gain in N-cadherin. In endometriosis disease, epithelial cells lose cell-cell adhesive strength and gain more mesenchymal characters [27]. This IUA process is similar to the development of adenomyosis [28]. Correspondingly, EMT regulates the mechanisms both in endometriosis and adenomyosis fibrosis development. As IUA is also a uterus fibrotic disease, a similar EMT mechanism may exist. From our study, E-cadherin and N-cadherin levels changed conversely in IUA endometrial epithelial cells. Epithelial cells stained negative for E-cadherin while starting to appear positive for N-cadherin, which is rarely observed in normal endometrium [29]. Furthermore, IUA epithelial cells gained another mesenchymal character; vimentin vividly appeared in gland epithelial cells. However, we also determined that the morphology of the vimentin-positive gland cells started to lose their columnar shape and looked like mesenchymal cells. In fact, we indicated that EMT does exist in the development of IUA.

As mentioned above, EMT is considered as an important step in fibrosis. And our study has showed E-cadherin's correlation with α -SMA. In fact, adding TGF- β 1 to endometrial epithelial and stromal cells changes the abilities of cell adhesion and differentiation and may ultimately lead to fibrosis [30, 31]. Actually, BMP7 has come into clinical use but it is never clarified in endometrial fibrous diseases, such as endometriosis, adenomyosis or IUA, etc. We firmly

believe that BMP7 play a significant role in IUA. Our study, for the first time, indicated the idea of BMP7 anti-fibrous function which may counteracts TGF- β 1-induced EMT. We are also interested in testing ER's role in IUA-L group, so we did IHC to explore its function. Luckily, we found ER expression decreased in IUA-L that denoted fibrous endometrium lost sensitivity to hormone stimulation.

Nowadays, more and more scientists introduce endometrial stem cell into uterus diseases. It seems endometrial stem cell is promising for IUA treatment and it may associate with IUA mechanism. While BMP family is also closely connected with stem cell, BMP7 may influence IUA coincident with endometrial stem cell. All in all, we believed that future researches would move to that way.

Conclusion

This research demonstrated Smad signaling in conjunction with EMT progression of IUA, which aimed to provide a new facet of IUA development. Overall, understanding the fibrotic mechanism remains a major challenge in IUA and definitely needs a further exploration.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant No. 81701398) and Shanghai Medical Center of Key Programs for Female Reproductive Diseases (No. 2017ZZ01016). The authors thank the Shanghai Key Laboratory of Female Reproductive Endocrine Related Diseases for providing us with such a good research platform.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Long Sui, Obstetrics and Gynecology Hospital of Fudan University, 419 Fangxie Road, Shanghai 200011, China. Tel: 0086-13801788118; E-mail: suilong@fudan.edu.cn

References

- [1] Takai IU, Kwayabura AS, Ugwa EA, Idrissa A, Obed JY and Bukar M. A 10-year review of the clinical presentation and treatment outcome of Asherman's syndrome at a center with limited resources. *Ann Med Health Sci Res* 2015; 5: 442-446.

Smad signaling coincides with EMT in IUA rat model

- [2] Hooker A, Fraenk D, Brolmann H and Huirne J. Prevalence of intrauterine adhesions after termination of pregnancy: a systematic review. *Eur J Contracept Reprod Health Care* 2016; 21: 329-335.
- [3] Hooker AB, Lemmers M, Thurkow AL, Heymans MW, Opmeer BC, Brolmann HA, Mol BW and Huirne JA. Systematic review and meta-analysis of intrauterine adhesions after miscarriage: prevalence, risk factors and long-term reproductive outcome. *Hum Reprod Update* 2014; 20: 262-278.
- [4] Chen Y, Liu L, Luo Y, Chen M, Huan Y and Fang R. Prevalence and impact of chronic endometritis in patients with intrauterine adhesions: a prospective cohort study. *J Minim Invasive Gynecol* 2017; 24: 74-79.
- [5] Yu D, Wong YM, Cheong Y, Xia E and Li TC. Asherman syndrome—one century later. *Fertil Steril* 2008; 89: 759-779.
- [6] Rockey DC, Bell PD and Hill JA. Fibrosis—a common pathway to organ injury and failure. *N Engl J Med* 2015; 373: 96.
- [7] Xu Q, Duan H, Gan L, Liu X, Chen F, Shen X, Tang YQ and Wang S. MicroRNA-1291 promotes endometrial fibrosis by regulating the ArhGAP29-RhoA/ROCK1 signaling pathway in a murine model. *Mol Med Rep* 2017; 16: 4501-4510.
- [8] Zhou Q, Wu X, Hu J and Yuan R. Abnormal expression of fibrosis markers, estrogen receptor alpha and stromal derived factor1/chemokine (C-X-C motif) receptor4 axis in intrauterine adhesions. *Int J Mol Med* 2018; 42: 81-90.
- [9] Salma U, Xue M, Ali Sheikh MS, Guan X, Xu B, Zhang A, Huang L and Xu D. Role of transforming growth factor-beta1 and smads signaling pathway in intrauterine adhesion. *Mediators Inflamm* 2016; 2016: 4158287.
- [10] Meng XM, Chung AC and Lan HY. Role of the TGF-beta/BMP-7/Smad pathways in renal diseases. *Clin Sci (Lond)* 2013; 124: 243-254.
- [11] Gelalis ID, Politis AN, Arnaoutoglou CM, Korompilias AV, Pakos EE, Vekris MD, Karageorgos A and Xenakis TA. Diagnostic and treatment modalities in nonunions of the femoral shaft: a review. *Injury* 2012; 43: 980-988.
- [12] Chen F, Guo QS, Chen LM, Jiang NH and Sui L. Effect evaluation of SD rat intrauterine adhesion model by whole layer removal of endometrium. *Fudan Xuebao (Yixueban)* 2018; 45: 376-383.
- [13] Salamonsen LA. Tissue injury and repair in the female human reproductive tract. *Reproduction* 2003; 125: 301-311.
- [14] Ning J, Zhang H and Yang H. MicroRNA326 inhibits endometrial fibrosis by regulating TGF-beta1/Smad3 pathway in intrauterine adhesions. *Mol Med Rep* 2018; 18: 2286-2292.
- [15] Ganjam VK and Evans TJ. Equine endometrial fibrosis correlates with 11beta-HSD2, TGF-beta1 and ACE activities. *Mol Cell Endocrinol* 2006; 248: 104-108.
- [16] Li J, Cen B, Chen S and He Y. MicroRNA-29b inhibits TGF-beta1-induced fibrosis via regulation of the TGF-beta1/Smad pathway in primary human endometrial stromal cells. *Mol Med Rep* 2016; 13: 4229-4237.
- [17] Li J, Du S, Sheng X, Liu J, Cen B, Huang F and He Y. MicroRNA-29b inhibits endometrial fibrosis by regulating the Sp1-TGF-beta 1/Smad-CTGF axis in a rat model. *Reprod Sci* 2016; 23: 386-394.
- [18] Liu F, Zhu ZJ, Li P and He YL. Creation of a female rabbit model for intrauterine adhesions using mechanical and infectious injury. *J Surg Res* 2013; 183: 296-303.
- [19] Xu XX, Cao LB, Wang Z, Xu Z, Zhang BQ, Wu SL, Qi SS, Yan L and Chen ZJ. Creation of a rabbit model for intrauterine adhesions using electrothermal injury. *J Zhejiang Univ Sci B* 2018; 19: 383-389.
- [20] Young VJ, Ahmad SF, Duncan WC and Horne AW. The role of TGF-beta in the pathophysiology of peritoneal endometriosis. *Hum Reprod Update* 2017; 23: 548-559.
- [21] Stoikos CJ, Harrison CA, Salamonsen LA and Dimitriadis E. A distinct cohort of the TGFbeta superfamily members expressed in human endometrium regulate decidualization. *Hum Reprod* 2008; 23: 1447-1456.
- [22] Li Q, Kannan A, Das A, DeMayo FJ, Hornsby PJ, Young SL, Taylor RN, Bagchi MK and Bagchi IC. WNT4 acts downstream of BMP2 and functions via beta-catenin signaling pathway to regulate human endometrial stromal cell differentiation. *Endocrinology* 2013; 154: 446-457.
- [23] Liu Y, Du SY, Ding M, Dou X, Zhang FF, Wu ZY, Qian SW, Zhang W, Tang QQ and Xu CJ. The BMP4-Smad signaling pathway regulates hyperandrogenism development in a female mouse model. *J Biol Chem* 2017; 292: 11740-11750.
- [24] Kodama A, Yoshino O, Osuga Y, Harada M, Hasegawa A, Hamasaki K, Takamura M, Koga K, Hirota Y, Hirata T, Takemura Y, Yano T and Taketani Y. Progesterone decreases bone morphogenetic protein (BMP) 7 expression and BMP7 inhibits decidualization and proliferation in endometrial stromal cells. *Hum Reprod* 2010; 25: 751-756.
- [25] Richards EG, El-Nashar SA, Schoolmeester JK, Keeney GL, Mariani A, Hopkins MR, Dowdy SC, Daftary GS and Famuyide AO. Abnormal uterine bleeding is associated with increased BMP7 expression in human endometrium. *Reprod Sci* 2017; 24: 671-681.

Smad signaling coincides with EMT in IUA rat model

- [26] Zeisberg M, Hanai J, Sugimoto H, Mammoto T, Charytan D, Strutz F and Kalluri R. BMP-7 counteracts TGF-beta1-induced epithelial-to-mesenchymal transition and reverses chronic renal injury. *Nat Med* 2003; 9: 964-968.
- [27] Bartley J, Juelicher A, Hotz B, Mechsner S and Hotz H. Epithelial to mesenchymal transition (EMT) seems to be regulated differently in endometriosis and the endometrium. *Arch Gynecol Obstet* 2014; 289: 871-881.
- [28] Shen M, Liu X, Zhang H and Guo SW. Transforming growth factor beta1 signaling coincides with epithelial-mesenchymal transition and fibroblast-to-myofibroblast transdifferentiation in the development of adenomyosis in mice. *Hum Reprod* 2016; 31: 355-369.
- [29] Poncelet C, Cornelis F, Tepper M, Sauce E, Magan N, Wolf JP and Ziol M. Expression of E- and N-cadherin and CD44 in endometrium and hydrosalpinges from infertile women. *Fertil Steril* 2010; 94: 2909-2912.
- [30] Yu L, Hu R, Sullivan C, Swanson RJ, Oehninger S, Sun YP and Bocca S. MFG8 regulates TGF-beta-induced epithelial mesenchymal transition in endometrial epithelial cells in vitro. *Reproduction* 2016; 152: 225-233.
- [31] Zhang Q, Duan J, Liu X and Guo SW. Platelets drive smooth muscle metaplasia and fibrogenesis in endometriosis through epithelial-mesenchymal transition and fibroblast-to-myofibroblast transdifferentiation. *Mol Cell Endocrinol* 2016; 428: 1-16.