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Expression of Transcriptional Repressor *Slug* Gene in Mouse Endometrium and its Effect During Embryo Implantation

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Abstract Slug, a member of the Snail family of zinc-finger transcription factors, is involved in regulating embryonic development and tumorigenesis. The aim of this study was to investigate the expression of Slug in mouse endometrium during early pregnancy and its possible role during embryo implantation. Fluorescence quantitative polymerase chain reaction and immunohistochemistry were applied to detect *Slug* mRNA and Slug protein expression in endometrium of nonpregnant and early pregnant mice, respectively. The expressions of *Slug* mRNA and its protein in pregnant group were higher than that in nonpregnant group and gradually increased from pregnancy day 1, reaching its maximum level on day 4 and then declining on days 5, 6, and 7. Immunohistochemistry showed that Slug protein was mainly present in luminal epithelium from pregnancy days 2 to 5 and in glandular epithelium from days 2 to 6 and enhanced significantly in stromal cells on days 4, 5, and 6. The number of embryos implanted was greatly decreased after Slug function in mouse endometrium was blocked by the intrauterine injection with anti-Slug polyclonal antibody on day 3 of pregnancy before implantation. These results suggested that up-regulation of Slug expression may play a key role in the embryo implantation in mice.

Keywords Embryo implantation · Expression study · Mouse endometrium · Slug

Introduction

The Snail superfamily of zinc-finger transcription factors, which comprise a highly conserved carboxyl terminal (including four to six zinc-finger domains) and an amino terminal, is involved in embryonic development and tumor progression. The Snail family includes snail, slug, and smuc [1]. Different family members play a role in many physiological or pathological processes such as embryogenesis, tumorigenesis, wound

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healing, apoptosis, left–right heterauxesis, the formation of appendages, neural differentiation, as well as the regulation of cell cycle and the determination of cell fate.

Slug, a member of the Snail family, specifically represses adherence junction components (E-cadherin and betacatenin), tight junction components (Occludin and ZO-1), and desmosomal junction components (Dsg2) and then obstructs the intercellular adhesion [2]. *Slug* gene has very conserved C2H2-type zinc-finger domain and can inhibit the transcription of target gene by linking to the specific E-boxes of its proximal promoter. Slug is necessary for gastrulation and mesoblastic formation [3, 4] and plays an important role in promoting cell migration including epithelial–mesenchymal transition (EMT) by suppressing several epithelial markers, adhesion molecules (including E-cadherin), and integrins (such as alpha3, beta1, and beta4) [5]. Many researches [2, 5–8] indicated that Slug is a repressor of the E-cadherin promoter and is involved in cell differentiation and apoptosis, and links to tumor progression and invasiveness. In addition, Slug up-regulation is followed with E-cadherin down-regulation and enhances the invasive ability, and this effect could be complemented by the presence of other EMT regulators (such as Snail, Twist, and SIP1) [9].

Embryo implantation, the first step of establishing successful pregnancy, is a crucial procedure for the reproduction of mammals. It requires delicate interactions between the embryos and the maternal uterine milieu. It will not be successful until embryonal invasion and endometrial receptivity achieve synchronization. And this key period is called the "window of receptivity" of implantation. Embryonal invasion and endometrial receptivity are concerned with the states of cell differentiation or development. There are two main phenotypes including epithelium and mesenchyme in the growth and development of cells, and they could carry out epithelial–mesenchymal transition. In EMT, the polarity structures of endometrial epithelial cells gradually disappear, and the expression of distinctive proteins (cadherin and vimentin) also begin to change, which promote the invasion of trophoblastic cells in the process of acquiring endometrium receptivity. However, the invasive ability of trophoblastic cells is regulated by extracellular matrix, matrix metalloproteinases, cell adhesion molecule, and other growth factors [10], just as well as the tumor cells.

The previous studies indicated that the embryo implantation process is paralleled with tumor invasion [10]. Although a role of Slug in tumor progression and invasion has been proposed and studied, whether this transcription factor plays distinct roles in embryo implantation has not been investigated and established. Hence, it is very significant to further investigate this important issue.

Materials and Methods

Animals

Depuratory National Institutes of Health mice, 6–8 weeks old and with body weight 25–30 g, were obtained from the Laboratory Animal Center of Chongqing Medical University in China [certificate: SCXK (YU) 2005002]. All animal procedures were approved by the Institutional Animal Care and Use Committee of Chongqing Medical University and in accordance with the policy of the Ethical Committee, State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences. Mice were caged in a controlled environment with a 14 h light:10 h dark cycle. According to vaginal smear, the estrus mice were chosen as nonpregnant group in this study (day 0 means nonpregnancy). Mature female mice were mated with fertile males of the same strain by caging together overnight (ratio=2:1) and then examined in the following morning. The pregnancy (day 1)

was confirmed by vaginal smears and/or the presence of a vaginal plug. Mice of nonpregnancy and pregnancy on days 1 to 7 were randomly divided into eight groups (day 0, day 1, day 2, day 3, day 4, day 5, day 6, and day 7), and there were 40 mice for each group. Twenty mouse uteri of each group were collected and stored in liquid nitrogen for fluorescence quantitative polymerase chain reaction (FQ-PCR), and the other 20 mouse uteri were fixed in 4% paraformaldehyde for immunohistochemistry. Another set of 40 pregnant mice on day 3 were randomly divided into two groups to undergo intrauterine injection for functional study of Slug, 20 mice in each group.

Reagents

Diaminobenzidine (DAB) color reagent box was purchased from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. (Beijing, China). Rabbit Anti-SLUG polyclonal Antibody was provided by Abcam Company (Cambridge, UK). FQ-PCR primers and fluorescent probes were designed and synthesized by Shanghai Genecore Company (Shanghai, China; Table 1). FQ-PCR analysis was performed on Applied Biosystems 7300 Real-Time PCR System.

FQ-PCR

RNA Extraction

The endometrium tissue was scraped and collected from mouse uterus. Total RNA was extracted from mouse endometrium according to the manufacturer's instructions of TRIzol reagent (Gibco, USA). The purity of total RNAs was examined by UV spectrophotometer. $A_{260}/A_{280} \geq 1.8$ was the standard. The integrity of the total RNA was identified by agarose gel electrophoresis. When the ratio of fluorescence intensity between 28s rRNA and 18s rRNA was 2:1, the molecule integrity of RNA was fine.

Reverse Transciption PCR

Total RNA was reverse-transcribed according to the procedure as follows: (1) RNA 5 μl (1 μg/μL), at 70 °C for 5 min, then iced bath for 1 min; (2) added Olig d(T) $_{18}$(TaKaRa) (500 ng/μL) 1 μl, 5× buffer 4 μl, ribonuclease inhibitor(TaKaRa) 1 μl, dNTPs (10 mmol/L) 2 μl, 0.1% diethyl pyrocarbonate-H_2O 6 μl, agitated and centrifuged, at 37 °C for 5 min, then iced bath for 1 min; (3) added Moloney murine leukemia virus reverse transcriptase (Promega) 1 μl, bulk volume 20 μl at 37 °C for 60 min, 70 °C for 10 min.

The primers of *Slug* and *GAPDH* were designed according to mouse *Slug* cDNA (123–266 bp, Genbank accession number NM_011415) and *GAPDH* cDNA (81-176 bp, Genbank accession number XM_001476723). The sequences of the primers were shown in

Table 1 Sequences of primers and fluorescent probes.

Slug	Forward primer	5′-GCTCCTTCCTGGTCAAGAAACAT-3′
	Reverse primer	5′-CCGAGGTGAGGATCTCTGGTT-3′
	Probe	FAM-ACGCCTCCAAGAAGCCCAACTACAGC-TAMRA
GAPDH	Forward primer	5′-GCACAGTCAAGGCCGAGAA-3′
	Reverse primer	5′-CCTCACCCCATTTGATGTTAGTG-3′
	Probe	FCATCACCATCTTCCAGGAGCGAGACCP

Table 1. A Blast search was performed to check the specificity of the DNA sequences of the primers. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was the interior control. The reaction conditions were 94 °C 5 min; 35 cycles for 94 °C 30 s, 58.2 °C 30 s, and 72 °C 40 s; 72 °C 10 min. The amplified fragments of *Slug* and *GAPDH* were 144 and 96 bp, respectively. The products were observed through 4% agarose gel electrophoresis, and 20 bp DNA ladder marker (TaKaRa) was used as a reference.

FQ-PCR

The starting copy number of unknown sample is acquired by standard curve in FQ-PCR, and the preparation of standard substance is the first step of making standard curve. Slug fragment was amplified in PCR meter, recovered and purified by cutting the gel, and then linked with PMD18-T carrier. After transformation and screening, the plasmid was amplified in competent bacteria, extracted and purified by shaking the germ, and carried out the sequencing. OD260 was detected by UV spectrophotometer, and the concentration and copy number of plasmid were calculated. The plasmid was diluted according to the way of tenfold serial dilution. Standard curve was made by using plasmid standard substance while running FQ-PCR of samples (Fig. 1a).

Quantification of the transcripts was carried out in the ABI 7300 Real-Time PCR System. The Taqman One-Step RT-PCR Master Mix Reagents was used at 25 μl tube as follows: Mg^{2+} (25 mmol/L) 3.0 μl, dNTPs (each 2.5 mmol/L) 2.0 μl, 10× buffer 2.5 μl, primer 1, 2 (20 μmol/L) each 0.5 μl, r*Taq* (5 U/μL) (TaKaRa) 0.4 μl, probe (20 μmol/L) 0.6 μl, cDNA template 2.0 μl. Amplification reaction conditions were 50 °C 2 min and 95 °C 10 min for one cycle and followed by 40 cycles of the amplification step (95 °C for 15 s, 58 °C for 30 s). Every specimen was detected by three parallel tubes. Briefly, the amounts of *Slug* and *GAPDH* mRNA in samples were estimated with standard curves representing the log of the input amount (log starting cDNA molecules) as the *X*-axis and the threshold cycle as the *Y*-axis (Fig. 1a). The signal detection plot of *Slug* mRNA was shown in Fig. 1b. The gene expression rate was obtained by normalizing the amount of *Slug* mRNA with that of *GAPDH.*

Immunohistochemistry

Mouse uteri were immediately cut into small pieces, fixed in 4% paraformaldehyde solution, dehydrated, and embedded in paraffin. Sections (4 μm) were cut, deparaffinized, and rehydrated. Endogenous peroxidase activity was inhibited by incubation with 3% H_2O_2 for 10 min and followed by a rinse in distilled water. For antigen retrieval, sections were placed in 10 mmol/L of citrate buffer, microwave hot repaired for 20 min, and naturally cooled at room temperature. After washing in phosphate-buffered saline (PBS) three times for 3 min each, nonspecific binding was blocked in 10% normal goat serum in PBS at 37 °C for 20 min. Then sections were incubated with rabbit antimouse Slug polyclonal antibodies (5 μg/mL, ab38551, Abcam Company, UK) in 10% goat serum overnight at 4 °C. In some sections, slug primary antibody was replaced with rabbit serum at the same dilution or concentration as a negative control. After incubation with biotinylated goat antirabbit IgG, the chromogenic reaction was carried out with DAB, and the reaction was terminated by tap water. Sections were counterstained with hematoxylin before permanent mounting and then evaluated under a light microscope. The degree of staining was assessed subjectively by blinded examination of the slides by two investigators. The image analysis of Slug protein expression in immunohistochemistry study was done by professional Image Analysis Software-ImagePro Plus 6.0.

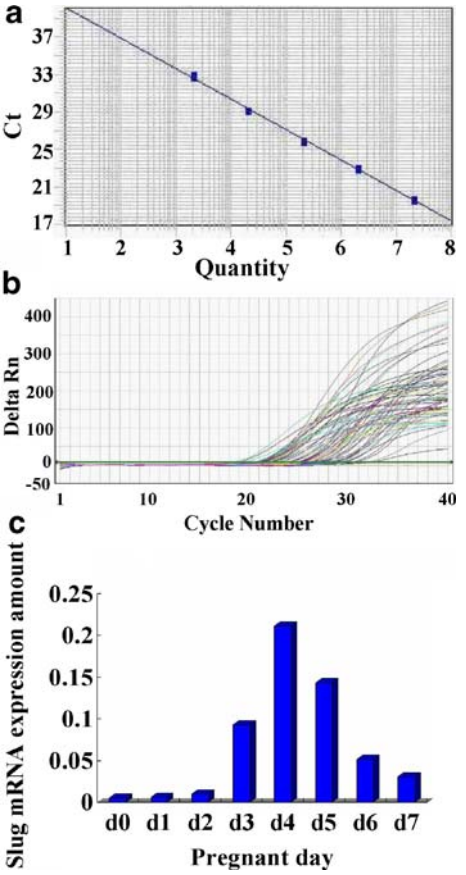

Fig. 1 FQ-PCR analysis of Slug mRNA in mouse endometrium during early pregnancy. **a** The standard curve plot of *Slug* gene (slope, −3.25808; *Y*-inter, 43.43292; R^2, 0.997905). *Standard curve* represents the log of the input amount (log starting cDNA molecules) as the *X-axis* and the threshold cycle as the *Y-axis*. **b** The signal detection figure of *Slug* represents the cycle number as the *X*-axis and the delta Rn as the *Y*-axis. **c** The mean plot of the relative value of *Slug*/*GAPDH* mRNA. This plot represents the day of pregnancy as the *X-axis* and the relative value of *Slug* mRNA/*GAPDH* mRNA as the *Y-axis*. *d0* nonpregnancy; *d1* day 1 of pregnancy, *d2* day 2 of pregnancy, *d3* day 3 of pregnancy, *d4* day 4 of pregnancy, *d5* day 5 of pregnancy, *d6* day 6 of pregnancy, *d7* day 7 of pregnancy

Function Blocking Studies of Slug During Embryo Implantation

Previous studies indicated that the window of implantation is very narrow, and implantation usually occurs on day 4 (day 1 = vaginal plug) in mouse [11]. To determine whether Slug plays a role during embryonic implantation, we blocked Slug function by intrauterine injection with anti-Slug polyclonal antibodies on day 3 of pregnancy before implantation. Forty pregnant mice on day 3 were randomly divided into two groups, 20 mice in each group. In the afternoon of day 3 of pregnancy, two uterus horns of each mouse were injected with sterile saline (5 μl) in control group; the right uterus horn of each mouse was injected with the anti-Slug polyclonal antibodies (5 μl, 5 μg/mL), and the left one was injected with sterile saline (5 μl) in treatment group. The number of embryos implanted was counted and recorded on day 8 of pregnancy to study the effects of Slug antibody on embryonic implantation in vivo. The data were analyzed by Student's *t* test.

Statistical Analyses

All data are presented as mean±SD and analyzed with SPSS 14.0 statistical software package. The statistical comparisons of relative mRNA expression of each gene were performed using one-way analysis of variance (ANOVA) with the Bonferroni–Dunnett's T3 post hoc multiple comparisons to examine for differences between every two experimental groups, and the difference was considered significant when p value <0.05.

Results

FQ-PCR Analysis of *Slug* mRNA in Mouse Endometrium

There were expressions of *Slug* in endometrium of pregnant and nonpregnant mice. The ratio of *Slug* mRNA/*GAPDH* mRNA in pregnant endometriun was higher than that in nonpregnant mouse. The results showed a gradual increasing trend as days passed, and the expression of *Slug* mRNA reached the maximum level on day 4 of pregnancy (Fig. 1c). One-way ANOVA showed that there were significant differences in the expression of *Slug* mRNA between days 3, 4, and 5 ($P<0.05$). But when day 0 was compared to day 1, day 3 compared to day 5, and day 6 compared to day 7, there were no significant differences ($P>0.05$), respectively (Table 2).

Immunohistochemical Analysis of Slug Protein Expression in Mouse Endometrium

Immunostaining of Slug protein expressed in mouse endometrium was showed in Fig. 2 and analyzed in Table 3. The positive expression of Slug was observed in the luminal and gland epithelium and stromal cells of pregnant mouse endometrium. Only faint signals were detected in nonpregnant mouse endometrium. Among the time points, less Slug protein was detected in the epithelium cells on day 0 and day 1, and the expression was hardly observed in stromal cells on day 0. Slug was located mainly in luminal epithelium from days 2 to 5 of pregnancy, in glandular epithelium from days 2 to 6 of pregnancy, and in stromal cells from days 3 to 6 of pregnancy. The strong immunoreactivity for Slug was observed in luminal epithelium, glandular epithelium, and stromal cells on day 4 of pregnancy. There was gradually increasing trend as days passed from day 2 to day 5, although there was no significant difference between days 4 and 5. However, Slug expression was decreased on

Table 2 Relative quantities of *Slug* mRNA expressed in the mouse endometrium on days 0–7 of pregnancy.

Day	d0*	d1**	d2***	d3****	d4*****	d5******	d6*******	d7*******
Slug/GAPDH	0.0056± 0.0025	0.0058± 0.0029	0.0106± 0.0032	0.0927± 0.0284	0.2112± 0.0394	0.1435± 0.0418	0.0515± 0.0213	0.0313± 0.0158

Numbers are means±SD, $n=20$, every specimen was performed in triplicate

d0 nonpregnancy, *d1* day 1 of pregnancy, *d2* day 2 of pregnancy, *d3* day 3 of pregnancy, *d4* day 4 of pregnancy, *d5* day 5 of pregnancy, *d6* day 6 of pregnancy, *d7* day 7 of pregnancy

*$P<0.05$ versus d2, d3, d4, d5, d6, d7, respectively; **$P<0.05$ versus d2, d3, d4, d5, d6, d7, respectively; ***$P<0.05$ versus d0, d1, d3, d4, d5, d6, d7, respectively; ****$P<0.05$ versus d0, d1, d2, d4, d6, d7, respectively; *****$P<0.05$ versus d0, d1, d2, d3, d6, d7, respectively; ******$P<0.05$ versus d0, d1, d2, d4, d6, d7, respectively; *******$P<0.05$ versus d0, d1, d2, d3, d4, d5, respectively; ********$P<0.05$ versus d0, d1, d2, d3, d4, d5, respectively

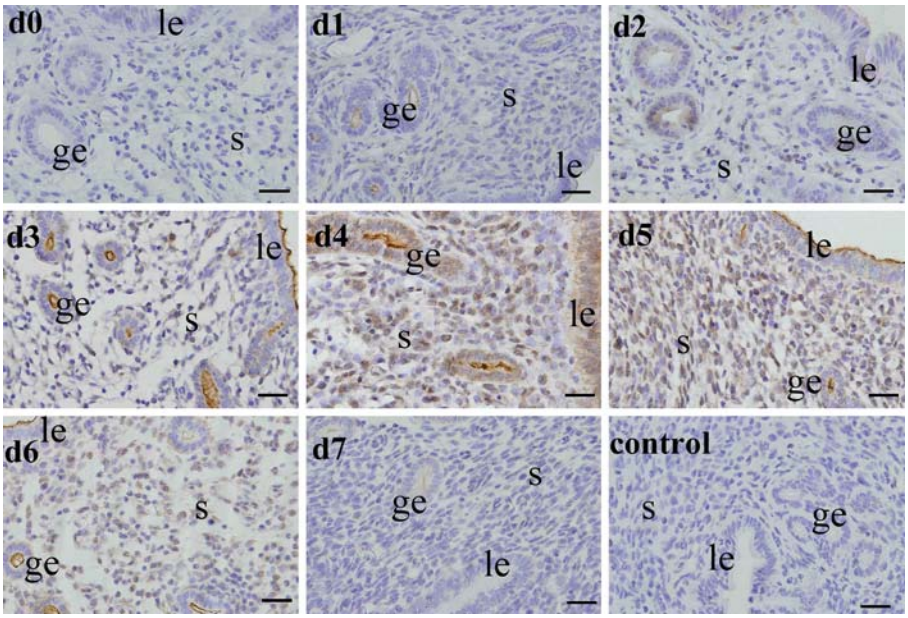

Fig. 2 Expression of Slug protein in mouse uterus at different time points during pregnancy by immunohistochemical analysis; *scale bar* 20.0 μm, *le* luminal epithelium, *s* stromal cell, *ge* glandular epithelium, *d1* first day of pregnancy, *d2* second day of pregnancy, *d3* third day of pregnancy, *d4* fourth day of pregnancy, *d5* fifth day of pregnancy, *d6* sixth day of pregnancy, *d7* seventh day of pregnancy, *d0* nonpregnancy, *control* negative control

Table 3 Qualitation and localization analysis of Slug protein expression in mouse endometrium.

Mouse endometrium	Expression of Slug protein at different time points							
	d0	d1	d2	d3	d4	d5	d6	d7
Luminal epithelium	±	±	+	+	++	+	+	±
Glandual epithelium	±	±	+	+	++	++	+	+
Stromal cell	−	±	±	+	++	++	+	±
Mean density of Slug protein	2.60E-05 ±3.64 E-06	2.70E-04 ±1.21 E-04	6.53E-04 ±1.22 E-04	3.26E-03 ±1.62 E-03	1.53E-02 ±3.02 E-04	5.92E-03 ±2.2 E-03	7.44E-04 ±3.54 E-04	3.90E-04 ±1.41 E-04

Numbers are means±SD, $n=20$

++ strongly positive, + positive, ± weakly positive, − negative, *d0* nonpregnancy, *d1* day 1 of pregnancy, *d2* day 2 of pregnancy, *d3* day 3 of pregnancy, *d4* day 4 of pregnancy, *d5* day 5 of pregnancy, *d6* day 6 of pregnancy, *d7* day 7 of pregnancy

days 6 and 7. Negative control did not produce any positive immunostaining signals for Slug protein.

Functional Analysis of Slug During Embryo Implantation

The results of uterus horn injection were shown in Fig. 3. The number of embryos implanted after intrauterine injection with anti-Slug antibodies was compared with control group. The total number of embryos implanted in treatment group was less than that in control group ($P<0.05$). Although the difference of embryos implanted between the left (6.9±1.1005) and the right (7.2±1.3984) horns in the control group was not significant, there was significant difference between the left (7.8±1.0328) horn and the right one (2.9± 1.1972) in the treatment group ($P<0.05$). Compared with the left horn, the number of embryos implanted in the right horn of the treatment group was obviously decreased ($P<$ 0.05).

Discussion

Our results indicated that the expressions of *Slug* mRNA and its protein in the pregnant group were higher than that in nonpregnant group, showed an increasing trend from pregnancy days 1 to 4, and reached the maximum on day 4 of pregnancy. The total number of embryos implanted was decreased by the intrauterine injection with anti-Slug polyclonal antibody on day 3 of pregnancy. These findings suggest that Slug might be involved in early pregnancy, but more investigations are necessary to validate this hypothesis.

The cell morphology, normal cell–cell contacts, programmed cell death, and the acquisition of invasive growth properties are influenced by the expression level of Slug [8]. In our study, the expression of *Slug* in early pregnant mice was higher than that in nonpregnant mice. Overexpression of Slug could suppress the expression of E-cadherin, promoted EMT [12], and increased the invasive ability of trophoblastic cells and the endometrium receptivity. Meanwhile, down-regulation of E-cadherin expression decreased cell junctional complex and influenced intercellular adhesion and conduction of signal and then promoted the invasion of embryo in parent endometrium. So we hypothesized that up-

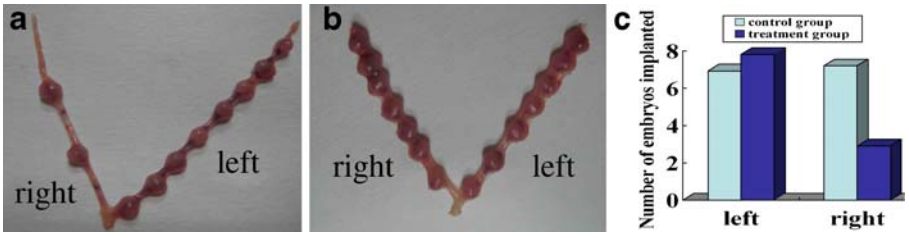

Fig. 3 Function-blocking studies using uterine horn injection of Slug antibody. **a** Treatment group. **b** Control group. **c** The number of embryos implanted was compared between left and right in control group and treatment group. Treatment group was injected with 5 μl (5 μg/mL) of anti-Slug polyclonal antibodies into the right horn of uteri and with 5 μl sterile saline into the left horn in the same mouse. Sterile saline (5 μl) was injected into two uterus horns in control group. The difference in embryos implanted between the left (6.9±1.1005) and the right (7.2±1.3984) horns in control group was not significant ($P>0.05$), but there was significant difference between the left (7.8±1.0328) horn and the right one (2.9±1.1972) in treatment group ($P<0.05$)

regulation of Slug expression played a role in successful embryo implantation by down-regulating the expression of E-cadherin.

Slug not only repressed E-cadherin but also down-regulated the expression of tight junction components (Claudin-1, Occludin, and ZO-1) [2, 13]. Tight junctions are responsible for establishing and maintaining epithelial cell polarity. The previous study had reported that overexpression of Slug could inhibit the expression of *Claudin-1* [14]. Hence, *Claudin-1* might be a direct downstream target gene of *Slug* in epithelial cells. The expression of Slug in our study gradually increased during pregnancy days 1–4 and reached the maximum level in "implantation window." On pregnancy days 2 and 3 before "implantation window," Slug was significantly increased in luminal epithelium compared with stroma. We hypothesized that Slug might be involved in destroying epithelial intercellular tight junction by altering epithelial cell polarity and morphology and then promoting embryo implantation.

According to Xing-hong MA et al. [15], Snail may play an important role during mouse embryo implantation. However, both Slug and Snail belong to the Snail family and have similar functions. They act to induce EMT and to be concerned with apoptosis [4]. In our study, high expressions of Slug mRNA and protein in stromal cells of mouse endometrium on days 4, 5, and 6 of pregnancy were observed. So we proposed Slug expression in the decidua may have a role for maintaining cell survival and preventing the formation of tight junctions so that embryos could invade into the decidua to establish a tight contact with maternal circulation.

Many secretions of endometrial glands are essential for uterine receptivity and successful implantation [16]. In our study, Slug was highly expressed in glandular epithelium of mouse endometria around the "implantation window," so it might play a role in secretory function during early pregnancy. In addition, Slug is involved in cell differentiation and apoptosis [2, 5–8], which play an important role in regulating decidualization of endometrium and invasion of trophoblastic cells [17]. Slug protein expression was intensively enhanced in mouse endometrium on day 3 of pregnancy and reached the highest value on day 4, the day of the "implantation window," [11] and remained elevated on day 5. These findings suggest that Slug might be involved in trophoblast invasion and decidualization of endometrium during implantation. Moreover, accumulated evidence showed that transforming growth factor (TGF)-β is an important regulatory factor during mouse embryo implantation [18] and might play an important role in the occurrence of apoptosis in the endometrium-trophoblast unit [19]. TGF-β induces Slug expression and mediates the repression of E-cadherin [20], so we suppose that Slug may play a part in embryo implantation.

In conclusion, we report here the expression of Slug in mouse endometria during early pregnancy. The high levels of Slug in mouse endometria around the "implantation window" raises the possibility that Slug could be involved in early pregnancy, especially embryo implantation. However, there were still a few implanted embryos when Slug signal was blocked, suggesting that Slug might not be the sole regulator during embryo implantation of mice. Further investigation is necessary to explore and define the function and molecular mechanism of Slug during embryo implantation in mice and determine whether this molecule may also be involved in implantation and infertility in humans, which is very significant in elevating the pregnancy rate of subsidiary reproduction technology.

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