HB-EGF regulates Prss56 expression during mouse decidualization via EGFR/ERK/EGR2 signaling pathway

Jie Liu¹, Fei Gao², Yue-Fang Liu¹, Hai-Ting Dou¹, Jia-Qi Yan¹, Zong-Min Fan¹ and Zeng-Ming Yang¹

¹College of Veterinary Medicine, South China Agricultural University, Guangzhou, China
²Department of Biology, Shantou University, Shantou, China

Abstract

Embryo implantation and decidualization are key steps for successful reproduction. Although numerous factors have been identified to be involved in embryo implantation and decidualization, the mechanisms underlying these processes are still unclear. Based on our preliminary data, Prss56, a trypsin-like serine protease, is strongly expressed at implantation site in mouse uterus. However, the expression, regulation and function of Prss56 during early pregnancy are still unknown. In mouse uterus, Prss56 is strongly expressed in the subluminal stromal cells at implantation site on day 5 of pregnancy compared to inter-implantation site. Under delayed implantation, Prss56 expression is undetected. After delayed implantation is activated by estrogen, Prss56 is obviously induced at implantation site. Under artificial decidualization, Prss56 signal is seen at the primary decidual zone at the initial stage of artificial decidualization. When stromal cells are induced for in vitro decidualization, Prss56 expression is significantly elevated. Dtgpr expression under in vitro decidualization is suppressed by Prss56 siRNA. In cultured stromal cells, HB-EGF markedly stimulates Prss56 expression through EGFR/ERK pathway. Based on promoter analysis, we also showed that Egr2 is involved in Prss56 regulation by HB-EGF. Collectively, Prss56 expression at implantation site is modulated by HB-EGF/EGFR/ERK signaling pathway and involved in mouse decidualization.

Introduction

Embryo implantation is the attachment of the embryo to the uterine luminal epithelium followed by invasion of embryos into the underlying stromal bed. Once embryos are implanted, uterine stromal cells will undergo decidualization (Hantak et al. 2014). Both embryo implantation and decidualization are synchronized and coordinated under the regulation of ovarian estrogen and progesterone (Wang & Dey 2006). However, the molecular mechanism underlying embryo implantation and decidualization is still unclear.

Proteolytic enzymes consist of over 2% of the known proteome and participate in many essential biological processes. The serine proteases constitute one of the largest families of proteolytic enzymes that are well recognized for their pivotal roles in physiological processes as diverse as development, digestion, coagulation, inflammation and immunity (Antalis et al. 2011). Serine proteinase is involved in the matrix degradation required for implantation (Salamonsen 1999). Trypsin-like serine protease is a kind of serine proteases possessing similar
activity as trypsin and specifically cleaved peptides with positively charged lysine or arginine (Evnin et al. 1990). Through activation of protease-activated receptors (PARs), trypsin-like serine proteases exert their roles among physiologic and pathologic processes (Hollenberg et al. 2014).

Among serine proteinases, implantation serine proteinase 1 (ISP1) encodes the embryo-derived enzyme strypsin, which is necessary for blastocyst hatching in vitro and the initiation of invasion. The ISP2 gene is expressed in endometrial glands and is regulated by progesterone during the peri-implantation period (O’Sullivan et al. 2004). Inhibition of ISP2 can significantly reduce the number of implantation sites in mice (Huang et al. 2004). Prss23 is strongly expressed in uterine luminal epithelium during mouse preimplantation period, and Prss35 is mainly expressed in subluminal stromal cells on days 5 and 6 of pregnancy (Diao et al. 2013).

Based on our preliminary microarray analysis on mouse uterus, Prss56 mRNA level at implantation site is significantly higher than that at inter-implantation site. Prss56 (protease, serine, 56, also called MCOP6 or Rik1700027L120) is a putative serine protease. Prss56 protein is predicted to be a secreted trypsin-like serine protease peptidase (Gal et al. 2011). However, the expression, regulation and function of Prss56 during early pregnancy are still unknown. In this study, we showed that Prss56 expression during decidualization is regulated by HB-EGF/EGFR/ERK/Egr2 signaling pathway.

**Materials and methods**

**Animals and treatment**

Mature CD1 mice used in this study were obtained from Hunan Slack Laboratory Animal Co., LTD. Mice were maintained under a specific pathogen-free (SPF) and controlled environment with a 12-h light:12-h darkness cycle. Animal experiment procedures were approved by the Animal Care and Use Committee of South China Agricultural University. Female mice were co-caged with fertile or vasectomized males in cages to induce pregnancy or pseudopregnancy. Day 1 of pregnancy or pseudopregnancy is the day of vaginal plug. Uteri at different time points were collected and flash-frozen for further analysis. Successful pregnancy was confirmed by flushing embryos from the uteri or oviducts from days 1 to 4. Tail intravenous injection with 0.1 mL of 1% Chicago blue dye (Sigma-Aldrich) was applied to identify implantation sites on days 5 and 6. The decidua can be visualized directly on days 7 and 8.

Models of artificial decidualization and delayed implantation were performed as previously described (Liang et al. 2014). Briefly, 10 μL of sesame oil (Sigma) was instilled into one uterine horn of female adults to induce deciduoma and the un-injected contralateral horn as control. Mice were killed on the day 5 or day 8 post vaginal plug. For delayed implantation, pregnant mice were ovariectomized on day 4 of pregnancy and subcutaneously injected with progesterone (P<sub>4</sub>, 1 mg/0.1 mL sesame oil/mouse; Sigma) from day 5 to 7. Delayed implantation was terminated by an injection of 25 ng/mouse estradiol-17β (E<sub>2</sub>, Sigma) on day 7. Mice were then killed on day 8. Implantation sites were identified by tail intravenous injection with Chicago blue 24 h after estrogen injection.

**Transfer of HB-EGF-soaked beads**

Affi-Gel blue Gel Beads (Bio-Rad, #1537301) of similar size with blastocyst were incubated with HB-EGF (heparin-binding EGF-like growth factor, 100 ng/μL) in 0.1% BSA/PBS under 37°C. After 1-h incubation, beads were washed with PBS at least three times. The HB-EGF-loaded beads (7–9 beads/horn) were transplanted into uterine lumen of day 4 pseudopregnant mice. Beads incubated with 0.1% BSA/PBS were transplanted into the other horn as control. Mice were killed for further analysis 24 h after the transplantation.

**In situ hybridization**

The cDNA fragment of each target gene was amplified with specific PCR primers (Table 1) and cloned into pGEM-T easy vector (Promega). After the direction of each insert ligated to the vector was verified, antisense or sense cRNA probes were synthesized in vitro by a digoxigenin RNA labeling kit (Roche Applied Science).

In situ hybridization was performed as previously described (Lei et al. 2012). Briefly, frozen sections were fixed in 4% PFA, hybridized for 16 h at 55°C, washed and incubated with sheep anti-digoxigenin antibody conjugated to alkaline phosphatase (1:5000, Roche) overnight. The signal was visualized with nitroblue tetrazolium (NBT, 0.4 mM) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, 0.4 mM) used as reaction substrates. Endogenous alkaline phosphatase activity was inhibited with 2 mM levamisole. Sections were counterstained with 1% methyl green.
Isolation and treatments of endometrium stromal cells

Endometrial stromal cells were isolated as previously described \( (\text{Lei et al., 2012}) \). Stromal cells were cultured with DMEM/F12 (Sigma) containing 10% (w/v) charcoal-stripped FBS (cFBS, Biological Industries, Beit Haemek, Israel). Cells were treated with HB-EGF (50 ng/mL, R&D Systems) for different time points. Correspondingly, cells treated with 1% BSA/PBS were regarded as the control. For the inhibition experiments, EGFR (EGF receptor) inhibitor (2 µM, Calbiochem, #364274) or ERK (extracellular regulated protein kinase) inhibitor U0126 (10 µM, Cell Signaling Technology, #9903S) were used in this study.

To induce stromal cells to undergo decidualization, \( \text{E}_2 \) combined with \( \text{P}_4 \) was used to treat cells for different time points, in the concentration of 10 nM or 1 µM, respectively. Since either \( \text{E}_2 \) or \( \text{P}_4 \) was dissolved in ethanol, cells treated with doubled volume of ethanol were served as the control.

Transfections

Stromal cells were transfected with siRNA against \( \text{Egr1} \) (Early growth factor 1), \( \text{Egr2} \) or \( \text{Prss56} \) expression (Guangzhou Ribo Co., Ltd, Guangzhou, China) using Lipofectamine2000 kit (Invitrogen). Methods for the transfection were performed according to the manufacturer’s protocol. After cells were transfected with \( \text{Prss56} \) siRNA for 48 h, stromal cells were induced for in vitro decidualization in DMEM/F12 containing 2% cFBS, \( \text{E}_2 \) (10 nM) and \( \text{P}_4 \) (1 µM) for 48 h. Hi GC siRNA was used as a negative control. The sequences used for RNA interference are provided in Table 1.

Western blot

Cultured cells were lysed in RIPA buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 1 mM Na\(_3\)VO\(_4\), 0.1% SDS, 1% Triton X-100, 1% Sodium deoxycholate) containing protease inhibitor cocktail (Roche). Denatured protein samples were subjected to SDS-PAGE, transferred onto polyvinylidene fluoride membranes (PVDF, Millipore) and probed with antibodies against p-ERK (extracellular regulated protein kinase) inhibitor U0126 (10 µM, Cell signaling Technology, #9903S) were used in this study.

To induce stromal cells to undergo decidualization, \( \text{E}_2 \) combined with \( \text{P}_4 \) was used to treat cells for different time points, in the concentration of 10 nM or 1 mM, respectively. Since either \( \text{E}_2 \) or \( \text{P}_4 \) was dissolved in ethanol, cells treated with doubled volume of ethanol were served as the control.

RNA extraction and real-time PCR

Total RNAs were extracted with TRIzol reagent (TaKaRa) and digested with RQI deoxyribonuclease I (Promega). Total RNAs (500 ng) were then reverse transcribed into cDNA with the 5x PrimeScript reverse transcriptase reagent kit (TaKaRa). Real-time PCR was performed with SYBR Premix Ex Taq kit (TaKaRa) on CFX96 TOUCHTM

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
<th>Accession no.</th>
<th>Size (bp)</th>
<th>Application</th>
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<td>( \text{Egr1} )</td>
<td>GAGCGAACAACCCCTATGAGGTCGGTTTGGCCTGGGATAA</td>
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<tr>
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Table 1 Primers used in this study.
Prss56 involvement in mouse decidualization

Statistical analysis

All the experiments were independently repeated at least 3 times. Data were processed using GraphPad Prism 6 Software. P values were calculated by Student’s unpaired test. Statistical significance is indicated by *P<0.05.

Results

Prss56 mRNA expression during early pregnancy

In situ hybridization was used to examine the mRNA expression of Prss56 during early pregnancy. Prss56 was undetected in mouse uteri from days 1 to 3. Signal was seen in the stromal cells under luminal epithelium on day 4.5. On day 5, Prss56 signal was strongly observed in the subluminal stromal cells at implantation site, while it was absent at inter-implantation site. On day 6, a weak signal of Prss56 was seen in the subluminal stromal cells. On days 7 and 8, there was no detectable signal (Fig. 1). There was no detectable signal when sense probe was used for in situ hybridization (data not shown).

In order to analyze whether Prss56 expression is dependent on the presence of blastocyst, Prss56 expression was examined under delayed implantation. Under delayed implantation, Prss56 was not detected. After delayed implantation was terminated by estrogen, Prss56 expression was strongly observed in the subluminal stromal cells at implantation site, similar to that on day 5 of pregnancy (Fig. 1).

Role of Prss56 during decidualization

In mouse uterus, uterine stromal cells will undergo decidualization following embryo attachment (Wang & Dey 2006). Additionally, decidualization can also be experimentally induced by mechanical or sesame oil stimuli (Herington et al. 2009). After sesame oil was intraluminally infused into one side of uterine horn on day 4 of pseudopregnancy, Prss56 signal was strongly detected in the subluminal stromal cells on day 5, but not seen on day 8 of pseudopregnancy. Furthermore, Prss56 signal was not seen in the un-injected uterine horn (Fig. 2A). Because Prss56 was strongly expressed in the primary decidua, we then examined Prss56 expression under in vitro decidualization. After mouse stromal cells were induced for in vitro decidualization, decidual/trophoblast...
prolactin-related protein (Dtprp), a marker for in vitro decidualization (Liang et al. 2010), was strongly expressed (Fig. 2B). Meanwhile, Prss56 was induced under in vitro decidualization even from 24 h (Fig. 2C). To further analyze the role of Prss56 during decidualization, Prss56 expression was knocked down by siRNA. After Prss56 level was suppressed by Prss56 siRNA (Fig. 2D and Supplementary Fig. 1, see section on supplementary data given at the end of this article), Dtprp expression was downregulated under in vitro decidualization (Fig. 2E).

HB-EGF regulation on Prss56 expression

In mice, HB-EGF signal is detected at the site of the blastocyst apposition 6–7 h before the attachment reaction (Das et al. 1994). HB-EGF-presoaked gelatin beads can induce implantation-like responses in uterine lumen of pseudopregnant mice (Paria et al. 2001). Therefore,
we asked whether Prss56 expression was regulated by HB-EGF. Signal of Prss56 expression can be seen in uteri of pseudopregnancy mice transplanted with beads incubated with HB-EGF but not in the control (Fig. 3A). On the other hand, after stromal cells were treated with HB-EGF, Prss56 expression was significantly induced from 1 h to 24 h after treatment (Fig. 3B). Meanwhile, Dtprrp expression was obviously increased from 1 h to 24 h after HB-EGF treatment, which was similar to Prss56 expression (Fig. 3C). Because HB-EGF is a ligand of EGFR (Lim & Dey 2009), stromal cells were treated with EGFR inhibitor to see whether HB-EGF stimulation on Prss56 is mediated by EGFR. The stimulation of HB-EGF on Prss56 was obviously abrogated by EGFR inhibitor (Fig. 3D). We have previously shown that HB-EGF regulates decidualization through ERK1/2 (Jiang et al. 2013). HB-EGF stimulation on Prss56 was also suppressed by ERK1/2 inhibitor (Fig. 3E). HB-EGF induction of p-ERK1/2 level was also abrogated by EGFR inhibitor (Fig. 3F). These data suggest that HB-EGF stimulates Prss56 expression via EGFR-ERK pathway.

Egr2 regulation on Prss56 expression

Based on our promoter analysis with JASPAR, there are Egr-binding sites in the Prss56 promoter (~2000 to 0bp). We previously showed that Egr1 is strongly expressed in the subluminal stromal cells at implantation site (Liang et al. 2014). At implantation site, Prss56 expression was markedly induced by the HB-EGF. Con, control. (G) Effects of EGFR inhibitor on HB-EGF-induced Egr2 expression. In the presence of EGFR inhibitor, Egr2 was downregulated. (H) Effect of ERK inhibitor on HB-EGF-induced Egr2 expression. Induction of Egr2 by HB-EGF was inhibited by ERK inhibitor. (I) HB-EGF stimulation on Prss56 was abrogated by Egr2 siRNA-1. (J) HB-EGF induction on Prss56 was abrogated by Egr2 siRNA-1. (K) HB-EGF induction on Dtprrp mRNA expression was inhibited by Egr2 siRNAData were presented as mean ± s.e.m. (n = 3).
was co-localized with Egr1. To test the effect of Egr1 and Egr2 on Prss56 expression, we knocked down Egr1 using siRNA. Although Egr1 level was reduced by Egr1 siRNA (Fig. 4A and Supplementary Fig. 1B), Prss56 expression was not affected by Egr1 knockdown (Fig. 4B). Because Egr2 is also highly expressed at implantation site on day 5 of pregnancy (Fig. 4E), and Egr1 and Egr2 share similar binding sites, we wondered whether Prss56 expression was regulated by Egr2. When Egr2 expression was knocked down by siRNA (Fig. 4C and Supplementary Fig. 1C), Prss56 expression was significantly downregulated (Fig. 4D and Supplementary Fig. 1C). When stromal cells were treated with HB-EGF, Egr2 expression was obviously increased (Fig. 4F). HB-EGF stimulation on Egr2 was also suppressed by EGFR inhibitor or ERK1/2 inhibitor (Fig. 4G and H). Under HB-EGF treatment, HB-EGF induction on both Prss56 and Dtprp expression was abrogated by Egr2 siRNA infection (Fig. 4I, J and K).

Discussion

In this study, Prss56 is strongly expressed in the subluminal stromal cells at implantation site. In vitro decidualization is suppressed by Prss56 siRNA. HB-EGF stimulates Prss56 expression via EGFR/ERK/Egr2 signaling pathway.

Prss56 encodes a protein containing 603 amino acids, showing similarity to trypsin-like serine proteases (Gal et al. 2011). Trypsin, an embryo-released serine protease, is required for embryo implantation through activation of epithelial Na+ channel and stimulation of COX-2 (Ruan et al. 2012). Both implantation serine proteinase ISP1 and 2 are serine proteinases essential for embryo hatching and implantation (Sharma et al. 2006). AEBSF, a general inhibitor of serine proteinases, can significantly inhibit embryo implantation in rats and attachment of mouse blastocysts on human epithelial cells (Jiang et al. 2011). Proprotein convertase 5/6 (PC6), a serine protease of the proprotein convertase (PC) family, is upregulated in the human endometrium specifically at the time of epithelial receptivity and stromal cell decidualization (Heng et al. 2011, Paule et al. 2015). High temperature requirement A4 (HTRA4) is a secreted serine protease highly expressed in the invasive extravillous trophoblasts that invade decidua. Htra1 and Htra3 interact with and degrade HtrA4 and thereby inhibit trophoblast-like JAR cell invasion (Chen et al. 2014). In our study, Prss56 is highly expressed in the primary decidual zone on day 5 of pregnancy, which is co-localized with COX-2 and mPGES-1, suggesting that Prss56 may regulate decidualization via stimulating COX-2 and mPGES-1 as trypsin does. Snail is also strongly expressed in the primary decidual zone (Ma et al. 2006), similar to Prss56 localization. We previously demonstrated that HB-EGF regulates snail expression through EGFR-ERK-Stat3 pathway (Jiang et al. 2013). However, the inhibitor of Stat3 has no effect on HB-EGF stimulation of Prss56 (data do not shown). At implantation site, Prss56 is co-localized with Egr1 (Liang et al. 2014). However, Prss56 expression is unaffected by Egr1 knockdown. Egr2 is another member of Egr family and highly expressed in primary decidual zone. Previous reports demonstrated that Egr2 knockout leads to a sharp decrease of Prss56 expression in boundary cap cells (Maro et al. 2004, Couplier et al. 2009). Similarly, our results have shown that Egr2 knockdown downregulates Prss56 expression in mouse uterus. Based on our data, HB-EGF induction on Prss56 expression should play a role during mouse decidualization.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-17-0636.

Declaration of interest

We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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