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

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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. Dtp 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.

Key Words

- ▶ Prss56
- ▶ decidualization
- ▶ uterus
- ▶ endometrium

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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 μ L 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 (P₄, 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-17 β (E₂, 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/ μ L) 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.

Table 1 Primers used in this study.

Gene	Primer sequences	Accession no.	Size (bp)	Application
<i>Egr1</i>	GAGCGAACAACCTATGAG GTCGTTTGGCTGGGATAA	NM_007913	102	Real-time PCR
siEgr1-1	CCAACAGTGGCAACACTTT			siRNA transfection
siEgr1-2	CCTTCGCTCACTCCACTAT			siRNA transfection
<i>Egr2</i>	CACCTCCTTCTACCCAT AACCTCCTATCACAACCTTC	NM_010118.3	433	<i>In situ</i> hybridization
<i>Egr2</i>	CTCCCGTATCCGAGTAGC GATGCCCGCACTCACAAT	NM_010118.3	144	Real-time PCR
siEgr2-1	GCTGCTATCCATAAGGTAT			siRNA transfection
siEgr2-2	GCATGATCAACATTGACAT			siRNA transfection
<i>Prss56</i>	ATTGCGGCGTGGAGTC AGGGTTGCTGCTGGTTCA	NM_027084	551	<i>In situ</i> hybridization
<i>Prss56</i>	GGCTGCACATTGCTTCG TGCGGGTCAAACCTTAGGG	NM_027084	132	Real-time PCR
siPrss56-1	GCCTCTGCAACAGAAGTTA			siRNA transfection
siPrss56-2	GCTTGGAACCTTCCACACT			siRNA transfection
<i>Dtprp</i>	AGCCAGAAATCACTGCCACT TGATCCATGCACCCATAAAA	NM_010088	119	Real-time PCR
<i>Rpl7</i>	GCAGATGTACCGCACTGAGATTC ACCTTTGGGCTTACTCCATTGATA	M29016	129	Real-time PCR
Negative control	CTCCGAACGTGTCACGT			siRNA transfection

Isolation and treatments of endometrium stromal cells

Endometrial stromal cells were isolated as previously described (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 μ M, Calbiochem, #364274) or ERK (extracellular regulated protein kinase) inhibitor U0126 (10 μ M, Cell signaling Technology, #9903S) were used in this study.

To induce stromal cells to undergo decidualization, E₂ combined with P₄ was used to treat cells for different time points, in the concentration of 10 nM or 1 mM, respectively. Since either E₂ or P₄ was dissolved in ethanol, cells treated with doubled volume of ethanol were served as the control.

Transfections

Stromal cells were transfected with siRNA against Egr1 (Early growth factor 1), Egr2 or 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 Prss56 siRNA for 48 h, stromal cells were induced for *in vitro* decidualization in DMEM/F12 containing 2%

cFBS, E₂ (10 nM) and P₄ (1 μ M) 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 Na₃VO₃, 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 (1:1000, Cell Signaling Technology, #4370), ERK (1:1000, Cell Signaling Technology, #4695) and α -Tubulin (1:1000, Cell Signaling Technology) diluted in 5% BSA (Sigma). Membranes were incubated in goat anti-rabbit antibody conjugated with horseradish peroxidase. The signals were developed in ECL Chemiluminescent kit (Thermo Fisher).

RNA extraction and real-time PCR

Total RNAs were extracted with TRIzol reagent (TaKaRa) and digested with RQ1 deoxyribonuclease I (Promega). Total RNAs (500 ng) were then reverse transcribed into cDNA with the 5 $\times$ PrimeScript reverse transcriptase reagent kit (TaKaRa). Real-time PCR was performed with SYBR Premix Ex Taq kit (TaKaRa) on CFX96 TOUCH™

(BIO-RAD). PCR conditions were as follows: 95°C for 3 min, followed by 39 cycles of 95°C for 10 s and 65°C for 5 s. All reactions were run in triplicate. $\Delta\Delta C_t$ analysis method was used to determine the relative changes of gene expression. Rpl7, a housekeeping gene, was used to normalize data. The primer sequences used for real-time PCR are listed in Table 1.

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

Figure 1

In situ hybridization of Prss56 in mouse uteri. During early pregnancy, Prss56 signal was strongly detected in the subluminal stromal cells on days 4.5 and 5 of pregnancy. Compared to delayed implantation, Prss56 expression was significantly induced after delayed implantation was activated by estrogen. Act, activation of delayed implantation; Del, delayed implantation; IS, implantation site; NI, non-implantation site. Scale bar = 300 μ m.

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

Figure 2

Prss56 mRNA expression and function during decidualization. (A) *In situ* hybridization of Prss56 mRNA expression under artificial decidualization. Prss56 was expressed on the subluminal stromal cells on the day 5 of artificial decidualization but not on day 8 and control horn. AD, artificial decidualization; AD-con, the un-injected contralateral horn. (B) Real-time PCR of Dtprp expression under *in vitro* decidualization from 6 h to 96 h. The significantly high expression of Dtprp indicates that the stromal cells were induced into decidualized cells. Con, control group; E+P, cells co-treated with E_2 and P_4 . (C) Real-time PCR of Prss56 mRNA expression under *in vitro* decidualization. Prss56 expression was significantly upregulated compared to the control from 24 h to 96 h. Con, control group; E+P, cells co-treated with E_2 and P_4 . (D) Real-time PCR of Prss56 expression after stromal cells under *in vitro* decidualization were treated with Prss56 siRNA. Prss56 expression was significantly downregulated by Prss56 siRNA. siPrss56-1, Prss56 siRNA-1. (E) Real-time PCR of Dtprp expression after stromal cells under *in vitro* decidualization were treated with Prss56 siRNA. Dtprp expression was significantly inhibited by Prss56 siRNA. E+P, Co-treatment with E_2 + P_4 . Data are presented as mean $\pm$ S.E.M. ($n=3$).

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,

Figure 3

HB-EGF induction on Prss56 expression through EGFR-ERK pathway. (A) *In situ* hybridization of Prss56 expression after HB-EGF-soaked beads were transferred into uterine lumen of pseudopregnant mice. Scale bar = 300 μ m. (B) Real-time PCR of Prss56 expression after stromal cells were treated with HB-EGF (50 ng/ μ L) at different time points. Cells were serum starved overnight with the medium containing 2% FBS before HB-EGF treatment. (C) Dtprp mRNA level after stromal cells were treated with HB-EGF (50 ng/ μ L) at different time points. (D) Effects of EGFR inhibitor (2 μ M) on HB-EGF-induced Prss56 expression. After serum starvation overnight, cells were pre-treated with the medium containing EGFR inhibitor for 1 h and then treated with HB-EGF and EGFR inhibitor for 6 h. (E) Effects of ERK inhibitor (10 μ M) on HB-EGF-induced Prss56 expression for 6 h. Cells were also pre-treated with ERK inhibitor for 1 h before HB-EGF treatment. (F) Western blot analysis of ERK and P-ERK proteins when stromal cells were treated with HB-EGF and EGFR inhibitor. Tubulin was used as a loading control. Data were presented as mean $\pm$ S.E.M. ($n=3$).

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, Dtrp 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

Figure 4

Egr2 involvement in HB-EGF regulation on Prss56 expression. (A) Egr1 mRNA level after stromal cells were treated with Egr1 siRNA-1. siEgr1-1, Egr1 siRNA-1. (B) Prss56 mRNA expression after stromal cells were treated with Egr1 siRNA-1. NC, negative control. (C) Egr2 mRNA level after stromal cells were treated with Egr2 siRNA-1. siEgr2-1, Egr2 siRNA-1. (D) Prss56 mRNA level after stromal cells were treated with Egr2 siRNA-1. (E) *In situ* hybridization of Egr2 expression at implantation sites in mouse uteri on days 4.5 and 5 of pregnancy. IS, implantation site. Scale bar = 300 μm. (F) HB-EGF induction on Egr2 expression. Egr2 expression in the stromal cells 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 Egr2 was reduced by Egr2 siRNA-1. (J) HB-EGF induction on Prss56 was abrogated by Egr2 siRNA-1. (K) HB-EGF induction on Dtrp mRNA expression was inhibited by Egr2 siRNA. Data 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 Dtpnp 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). AEBSE, 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 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, Coudrier *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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