Differential expression and regulation of *Cryab* in mouse uterus during preimplantation period

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Abstract

The aim of this study was to examine the expression and regulation of the crystallin, alpha B (*Cryab*) gene in mouse uterus during the peri-implantation period by *in situ* hybridization and real-time PCR. There was no detectable *Cryab* mRNA signal on days 1–4 of pregnancy. On day 5 of pregnancy when embryo implanted, a high level of *Cryab* mRNA signal was found in the subluminal stroma surrounding the implanting blastocyst. On days 6–8, *Cryab* mRNA was strongly expressed in the primary decidua. By real-time PCR, a high level of *Cryab* expression was detected on days 7 and 8 of pregnancy, although *Cryab* expression was seen from days 1 to 8. Under *in vivo* and *in vitro* artificial decidualization, *Cryab* expression was significantly elevated. Compared with the progesterone-primed delayed implantation uterus, a high level of *Cryab* mRNA expression was observed in estrogen-activated implantation uterus. In the uterine stromal cells, cAMP, estrogen, and progesterone could induce the expression of *Cryab* gene. In the ovariectomized mouse uterus, estrogen could also induce the expression of *Cryab* while progesterone inhibited its expression. Our data suggest that *Cryab* may play an important role during mouse embryo implantation and decidualization and that estrogen and progesterone can regulate the expression of *Cryab* gene.

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Introduction

Embryo implantation is a critical initial step in the establishment of a successful pregnancy (Cakmak & Taylor 2011). Accumulating data have shown that heat-shock proteins (HSPs), a family of highly conservative multifunctional polypeptides that are classified into HSPH1 (HSP105), HSP90, HSPD1 (HSP60), and other small HSPs according to molecular weight, are closely related to embryo implantation and decidualization (Neuer *et al.* 2000, Yuan *et al.* 2009). During the menstrual cycle, HSP90 and HSP70 were expressed in human endometrium (Tabibzadeh & Broome 1999, Neuer *et al.* 2000). Injection of antisense oligodeoxynucleotides to HSPH1 into the rat uterine horn could obviously reduce the number of implanted embryos (Yuan *et al.* 2009). In addition, small HSPs have been thought to play a role in decidualization (Bany & Schultz 2001).

Alpha-crystallins, the principal members of small HSP family, comprise two types of highly homologous subunits, crystallin, alpha A (CRYAA, also known as HSPB4), and crystallin, alpha B (CRYAB, also known as

HSPB5), which are ~60% identical in amino acid sequence and are encoded by two separate, unlinked genes (Robinson & Overbeek 1996, Brady *et al.* 1997). CRYAA is a lens-specific protein that could protect proteins from aggregation in the eye lens, while CRYAB which was originally discovered in the mammalian eye lens as the B-subunit of α -crystallin is also expressed in a number of non-lenticular tissues, including the heart, skeletal muscles, kidney, lung, brain, placenta, and uterus (Robinson & Overbeek 1996, Gruidl *et al.* 1997, Mineva *et al.* 2008, Mainz *et al.* 2012). In term human placental tissues, CRYAB was visibly observed in the stromal cells of the placental villi (Mineva *et al.* 2008). In equine endometrium, CRYAB was detected on days 12 and 13.5 of pregnancy (Klein *et al.* 2010, Merkl *et al.* 2010). During the menstrual cycle, CRYAB was expressed in human and baboon endometrium and also upregulated during the window of implantation (Gruidl *et al.* 1997). According to our (unpublished) microarray data, *Cryab* expression was significantly higher in mouse uterus at implantation sites rather than at interimplantation sites. However, the expression and regulation of *Cryab* in mouse uterus during implantation

and decidualization has not been defined so far. Thus, this study was undertaken to examine the expression and regulation of *Cryab* gene in mouse uterus during the peri-implantation period by *in situ* hybridization and real-time PCR.

Results

Cryab mRNA expression during early pregnancy

In situ hybridization was used to examine the spatial distribution of *Cryab* mRNA expression in mouse uterus (Fig. 1A). There was no detectable *Cryab* mRNA signal on days 1–4 of pregnancy. On day 5 of pregnancy, a high level of *Cryab* mRNA signal was found in the subluminal stroma immediately surrounding the implanting blastocyst, whereas no signal was detected in the inter-implantation area. From days 6 to 8, *Cryab* mRNA signal was detected in the primary decidua at a high level and gradually increased as the decidua developed. No *Cryab* signals were seen in the embryos from days 5 to 8 of pregnancy by *in situ* hybridization.

To verify *Cryab* mRNA expression, real-time PCR was also performed. A high level of *Cryab* expression was detected on days 7 and 8 of pregnancy, although *Cryab* expression was seen from days 1 to 8 (Fig. 1B). Compared with the interimplantation sites, *Cryab* mRNA

expression was higher at implantation sites on day 5 of pregnancy (Fig. 1C).

Cryab mRNA expression during pseudopregnancy

To address whether *Cryab* expression was dependent on embryos, we examined the expression of *Cryab* during pseudopregnancy. *In situ* hybridization results showed that there was no detectable *Cryab* mRNA signal in the uteri from days 1 to 5 of pseudopregnancy (Fig. 2A, B, C, and D). However, *Cryab* expression was relatively high on day 3 of pseudopregnancy by real-time PCR, although *Cryab* expression was detected from days 1 to 5 (Fig. 3A).

Cryab mRNA expression during delayed implantation and activation

In order to see whether *Cryab* expression was dependent on activation status of blastocyst, we examined its expression in the delayed-implantation uterus or activated implantation sites. Under delayed implantation, *Cryab* mRNA was not detected in mouse uterus (Fig. 2E). Once delayed implantation was terminated by estrogen treatment, *Cryab* mRNA signal was detected in the subluminal stromal cells surrounding the implanting embryo (Fig. 2F). Simultaneously, a significantly high

Figure 1 *Cryab* expression during early pregnancy. (A) *In situ* hybridization of *Cryab* expression in mouse uteri during early pregnancy on days 1, 2, 3, 4, 5, 6, 7, and 8. No hybridization signals were seen in mouse uterus on day 8 of pregnancy when DIG-labeled *Cryab* sense probe was used to replace the antisense probe as a negative control. Asterisks indicate embryo Bar=60 μ m. (B) Real-time PCR analysis of *Cryab* expression in mouse uterus on days 1–8 during pregnancy. (C) Real-time PCR analysis of *Cryab* expression at the implantation sites (5-I) and interimplantation sites (5-N) on day 5 of pregnancy. Data are shown as mean $\pm$ S.E.M. Bars with different letters at the top differ significantly.

Figure 2 *In situ* hybridization of *Cryab* expression in mouse uteri during pseudopregnancy on days 1 (A), 3 (B), 4 (C), and 5 (D), delayed implantation (E) and activation (F), artificial decidualization (H), and contralateral uninjected uterine horn (I). No hybridization signals were seen in mouse uteri under artificial decidualization when DIG-labeled *Cryab* sense probe was used for *in situ* hybridization (G). Asterisks indicate embryo. Bar = 60 μ m.

level of *Cryab* expression was detected in activated implantation uterus compared with the delayed uterus by real-time PCR (Fig. 3B).

***Cryab* expression under artificial decidualization**

Because *Cryab* was strongly expressed in the decidua during early pregnancy, artificially induced decidualization was performed to see whether *Cryab* expression in the decidua was dependent on the presence of embryos. Under artificial decidualization, *Cryab* mRNA signal was strongly detected in the decidualized cells (Fig. 2H). However, no visible *Cryab* mRNA signal was found in uninjected control uterus (Fig. 2I). By real-time PCR analysis, *Cryab* expression was also significantly upregulated in the decidualized uterus compared with the control uterus (Fig. 3C).

***Cryab* expression under *in vitro* decidualization**

Primary stromal cells isolated from mouse uteri on day 4 of pregnancy were treated with a combination of estrogen and progesterone to induce *in vitro* decidualization. Because prolactin family 8, subfamily a, member 2 (*Prl8a2*) is a reliable marker for decidualization in mice, we examined whether *Prl8a2* was expressed under *in vitro* decidualization. By real-time PCR analysis, the expression of *Prl8a2* was significantly upregulated from 24 to 96 h of culture (Fig. 4A), indicating that the method inducing *in vitro* decidualization was successful. Likewise, *Cryab* expression was also significantly elevated from 24 to 96 h after *in vitro* decidualization (Fig. 4B).

cAMP regulation on *Cryab* expression

Because cAMP is a key player in decidualization, the time course of *Prl8a2* and *Cryab* expression was examined at 1, 3, 6, 12, and 24 h after the cultured uterine stromal cells were treated with 8-bromoadenosine-cAMP (8-Br-cAMP, Sigma). The results showed that *Cryab* expression was significantly higher at 6 h, although 8-Br-cAMP could induce *Cryab* expression from 3 to 24 h of culture (Fig. 5B). Simultaneously, *Prl8a2* expression gradually increased and reached the peak level at 24 h after 8-Br-cAMP treatment (Fig. 5A).

Steroid hormonal regulation on *Cryab* expression

As estrogen and progesterone are essential for mouse embryo implantation, ovariectomized mice were used to examine the effects of steroid hormones on *Cryab* expression. A low level of hybridization signal was seen in the uterus of ovariectomized mice (Fig. 6). Progesterone had no effect on *Cryab* expression, while estrogen could slightly induce the expression of *Cryab* mRNA in the luminal and glandular epithelium at 12 h (Fig. 6). By real-time analysis, *Cryab* mRNA expression increased in ovariectomized mice uteri after injection of estrogen and reached the highest level at 12 h (Fig. 7A). However, the upregulation of estrogen was significantly blocked by a pretreatment with ICI 182 780 (Fig. 7D). In contrast, progesterone injection resulted in a decline in uterine *Cryab* mRNA levels, which reached a nadir by 24 h (Fig. 7B). A significantly higher level of *Cryab* expression was detected in the uterus of ovariectomized mice that had received injections of progesterone at 1 h

Figure 3 *Cryab* expression during pseudopregnancy, delayed and activated implantation, and artificial decidualization. (A) Real-time PCR analysis of *Cryab* expression in mouse uterus on days 1–5 during pseudopregnancy. (B) Real-time PCR analysis of *Cryab* expression in mouse uterus during delayed implantation and activation. (C) Real-time PCR analysis of *Cryab* expression under artificial decidualization. Data are shown as mean $\pm$ S.E.M. Bars with different letters at the top differ significantly.

later after pretreatment of RU486 compared with that of progesterone treatment only (Fig. 7E). Additionally, a low level of *Cryab* expression was observed at 24 h after a combined injection of estrogen and progesterone (Fig. 7C).

In the *in vitro*-cultured stromal cells, *Cryab* mRNA expression was significantly induced at 24 h after treatment with estrogen or progesterone (Fig. 7F and G). ICI 182 780 could significantly reduce estrogen-stimulated *Cryab* expression (Fig. 7F). A similar result was also observed with RU486 treatment (Fig. 7G).

Discussion

In this study, the expression and regulation of *Cryab* in mouse uterus during early pregnancy were examined by *in situ* hybridization and real-time PCR. Our results showed that *Cryab* mRNA signal from *in situ* hybridization was not detected in the mouse uteri from days 1 to 4 of pregnancy. Likewise, *Cryab* was also not seen in any of the proliferative endometrium from humans and baboons (Gruidl *et al.* 1997). However, *Cryab* mRNA

was detected in mouse uterus using real-time PCR. The reason might lie in different sensitivity between real-time PCR and *in situ* hybridization. On day 5 of pregnancy when blastocysts implanted, a high level of *Cryab* mRNA signal was found in the subluminal stroma at implantation sites. No corresponding signals were observed at the interimplantation sites on day 5 of pregnancy or during pseudopregnancy. Simultaneously, a high level of *Cryab* expression was also detected in activated implantation uteri but not in delayed uteri. These results indicated that *Cryab* might play an important role in the process of mouse implantation and was influenced by the active blastocyst. In human endometrium, *Cryab* expression is upregulated during the window of implantation (Gruidl *et al.* 1997), which further supported a role for *Cryab* in implantation. Additional studies have found that *Cryab* could regulate angiogenesis by modulating the expression of vascular endothelial growth factor (VEGF), which was the main factor responsible for increased endometrial vascular permeability at implantation (Rabbani & Rogers 2001, Rockwell *et al.* 2002, Kase *et al.* 2010, Ruan *et al.* 2011). Inhibition of VEGF could significantly reduce the number of implantation sites (Rabbani & Rogers 2001, Rockwell *et al.* 2002, Guo *et al.* 2012). However, whether *Cryab* was capable of regulating VEGF in the

Figure 4 Real-time PCR analysis of *Prl8a2* (A) and *Cryab* (B) expression *in vitro* decidualization of uterine stromal cells. Data are shown as mean $\pm$ S.E.M. *Statistical significance ($P < 0.05$); E, estrogen; P, progesterone.

Figure 5 Effects of 8-Br-cAMP on *Prl8a2* (A) and *Cryab* (B) expression in uterine stromal cells. Data are shown as mean $\pm$ S.E.M. *Statistical significance ($P < 0.05$).

process of mouse implantation remained to be determined, although *Cryab* and VEGF are expressed in the subluminal stroma surrounding the implanting blastocyst (Chakraborty *et al.* 1995). Additionally,

Cryab could also regulate the expression of *Trp53* (*p53*) gene (Watanabe *et al.* 2009). In *Trp53*-deficient female mice, the number of implantation sites was significantly reduced due to impaired uterine leukemia inhibitory factor (*Lif*) gene expression, which was critical for blastocyst implantation (Wang & Dey 2006, Hu *et al.* 2007). Thus, we speculated that *Cryab* might play a role in the process of mouse implantation through influencing the expression of *Trp53* and *Lif* genes.

Uterine stromal cell decidualization is integral to successful embryo implantation (Das 2009). Our results showed that *Cryab* mRNA signal was detected strongly in the decidua on days 6–8 of pregnancy using real-time PCR and *in situ* hybridization. Likewise, the high level of *Cryab* mRNA could be detected in decidualized cells under artificial decidualization, and in the stromal cell cultures that were induced for *in vitro* decidualization. These results suggest that *Cryab* may play an important role during decidualization. A previous study found that cAMP levels significantly increased after sesame oil stimulation of the uterine horn (Rankin *et al.* 1977). Moreover, cAMP could induce and regulate the decidualization of uterine stromal cells (Gellersen & Brosens 2003, Liang *et al.* 2010). In mouse uterine stromal cells cultured *in vitro*, we found that cAMP induced the expression of the *Cryab* gene, which further supports a role for *Cryab* in decidualization. Further study found that *Cryab* could promote angiogenesis during tube morphogenesis (Dimberg *et al.* 2008). Angiogenesis is crucial for decidualization because administration of AGM-1470 (a nonspecific angiogenesis

Figure 6 *In situ* hybridization of *Cryab* expression in ovariectomized mouse uteri after injection of estrogen, progesterone, or a combination of estrogen and progesterone for 0 (control), 1, 3, 6, 12, and 24 h. Bar = 60 μ m. E, estrogen; P, progesterone.

Figure 7 Hormonal regulation of *Cryab* expression. (A) Real-time PCR analysis of *Cryab* expression in ovariectomized mouse uterus after estrogen treatments for 0, 1, 3, 6, 12, and 24 h. (B) *Cryab* expression in ovariectomized mouse uterus after injection of progesterone. (C) *Cryab* expression in ovariectomized mouse uterus after injection of estrogen plus progesterone. (D) *Cryab* expression in ovariectomized mouse uterus after injection of estrogen and ICI 182 780. (E) *Cryab* expression in ovariectomized mouse uterus after injection of progesterone and RU486. (F) *Cryab* expression after stromal cells were treated with estrogen, or both estrogen and ICI 182 780. (G) *Cryab* expression after stromal cells were treated with progesterone, or both progesterone and RU486. Data are shown mean $\pm$ S.E.M. Bars with different letters at the top differ significantly.

inhibitor) can result in impaired decidualization (Klauber *et al.* 1997, Guo *et al.* 2012). These data indicate that *Cryab* may direct uterine angiogenesis during decidualization.

Embryo implantation and decidualization are closely regulated by the ovarian estrogen and progesterone (Singh *et al.* 2011). On day 5 of pregnancy when the level of maternal estrogen was high, *Cryab* mRNA was highly expressed in mouse uterus, indicating that estrogen might be implicated in regulating the expression of *Cryab* gene. In the *Cryab* promoter region, there were multiple half-site estrogen-responsive elements (Frederikse *et al.* 1994, Gruidl *et al.* 1997). In this study, we also found that estrogen could upregulate the expression of *Cryab* mRNA in ovariectomized mouse uterus and uterine stromal cells. Moreover, the upregulation could be antagonized by

estrogen receptor antagonist ICI 182 780, suggesting estrogen receptor requirement for this induction. In contrast, progesterone could inhibit *Cryab* expression through progesterone receptor in ovariectomized mouse uterus. In the uterine stromal cells cultured *in vitro*, *Cryab* expression was induced by progesterone. The discrepancies suggested that *Cryab* was also expressed in the other cell constituents of uterus where progesterone might inhibit *Cryab* expression. Indeed, *Cryab* has also been observed in the endometrial epithelial cells and muscle cells (Gruidl *et al.* 1997, Sanbe 2011). However, it is still to be determined whether progesterone could inhibit the expression of *Cryab* in the cells.

In conclusion, *Cryab* may be important for mouse embryo implantation and decidualization and regulated by estrogen and progesterone.

Materials and Methods

Animals

Matured mice (Kunming White strain) were caged in a controlled environment with a cycle of 14 h light:10 h darkness. All animal procedures were approved by the Institutional Animal Care and Use Committee of Jilin University. To confirm reproducibility of results, at least three mice per group were used in each stage or treatment in this study.

Pregnancy and pseudopregnancy

Adult female mice were mated with fertile or vasectomized males of the same strain to induce pregnancy or pseudopregnancy by co-caging respectively (day 1 = day of vaginal plug). On days 1–4, pregnancy was confirmed by recovering embryos from the oviducts or uterus. The implantation sites on day 5 were identified by i.v. injection of 0.1 ml 1% Chicago blue (Sigma) in 0.85% sodium chloride.

Delayed implantation and activation

To induce delayed implantation, pregnant mice were ovariectomized under ether anesthesia at 0830–0900 h on day 4 of pregnancy. Progesterone (1 mg/mouse; Sigma) was injected subcutaneously to maintain delayed implantation from days 5 to 7. Estrogen (25 ng/mouse, Sigma) was given to progesterone-primed delayed-implantation mice to activate blastocyst implantation. The mice were killed to collect uteri 24 h after estrogen treatment. The implantation sites were identified by i.v. injection of Chicago blue solution. Delayed implantation was confirmed by flushing the blastocysts from the uterus.

Artificial induced decidualization

Artificial decidualization was induced by intraluminally infusing 25 μ l sesame oil into one uterine horn on day 4 of pseudopregnancy, while the contralateral uninjected horn served as a control. The uteri were collected on day 8 of pseudopregnancy. Decidualization was confirmed by weighing the uterine horn and by histological examination of uterine sections.

Steroid hormonal treatments

Mature female mice were ovariectomized and, after 2 weeks, given a single s.c. injection of estrogen (100 ng/mouse), progesterone (2 mg/mouse), or a combination of the same doses of progesterone and estrogen (Guo *et al.* 2012). Uteri were then collected 1, 3, 6, 12, and 24 h after steroid treatment. To examine whether nuclear receptors for estrogen or progesterone are involved in steroid hormonal regulation, ovariectomized mice were injected with ICI 182 780 (an estrogen receptor antagonist) or RU486 (a progesterone receptor antagonist) 1 h before estrogen or progesterone injection respectively. ICI 182 780 and RU486 were injected at a dose of 500 μ g and 1 mg per mouse respectively (Guo *et al.*

2012). All steroids and antagonists were dissolved in sesame oil and injected subcutaneously. Controls received the vehicle only (0.1 ml/mouse).

In situ hybridization

Total RNAs from the mouse uteri were reverse transcribed and amplified with *Cryab* primers. *Cryab* forward primer 5'-GGTCTGGGGGACGTGATTG and reverse primer 5'-CAG-CAGGCTTCTCTTCACGG were designed based on the sequence of the *Mus musculus* crystallin, alpha B gene (GenBank accession number NM_009964). The amplification of *Cryab* cDNA was performed with 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. The amplified fragment (230 bp) of *Cryab* was recovered from the agarose gel and cloned into pGEM-T plasmid (pGEM-T Vector System 1, Promega). The orientation of the *Cryab* fragment in the pGEM-T plasmid was determined by PCR using a combination of the primers for T7, SP6, and mouse *Cryab*. The cloned *Cryab* fragment was further verified by sequencing. *Cryab*-containing plasmid was amplified with the primers for T7 and S6 to prepare templates for labeling. Digoxigenin (DIG)-labeled antisense and sense cRNA probes were transcribed *in vitro* using a DIG RNA labeling kit (Roche Diagnostics GmbH).

Frozen sections (10 μ m) were mounted on 3-amino-propyltriethoxysilane (Sigma)-coated slides and fixed in 4% paraformaldehyde solution in PBS. The sections were washed in PBS twice, treated with 1% Triton-100 for 20 min, and washed again in PBS three times. Following the prehybridization in the solution of 50% formamide and 5 $\times$ SSC (1 $\times$ SSC is 0.15 M sodium chloride, and 0.015 M sodium citrate) at room temperature for 15 min, the sections were hybridized in the hybridization buffer (5 $\times$ SSC, 50% formamide, 0.02% BSA, 250 mg/ml yeast tRNA, 10% dextran sulfate, 1 mg/ml denatured DIG-labeled antisense, or sense RNA probe for mouse *Cryab*) at 55 °C for 16 h. After hybridization, the sections were washed sequentially in 50% formamide/5 $\times$ SSC at 55 °C for 15 min, 50% formamide/2 $\times$ SSC at 55 °C for 30 min, 50% formamide/0.2 $\times$ SSC at 55 °C twice for 30 min each, and 0.2 $\times$ SSC at room temperature for 5 min. After nonspecific binding was blocked in 1% block reagent (Roche) for 1 h, the sections were incubated in sheep anti-DIG antibody conjugated with alkaline phosphatase (1:5000, Roche) in 1% block reagent overnight at 4 °C and then washed in buffer I (100 mM Tris-HCl, pH 7.5, and 150 mM NaCl) three times for 5 min each before equilibration in Buffer II (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 50 mM MgCl₂) for 5 min. Endogenous alkaline phosphatase activity was inhibited with 2 mM levamisole (Sigma). The signal was visualized with 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate and 0.4 mM nitroblue tetrazolium in buffer II. All the sections were counterstained with 1% methyl green in 0.12 M glacial acetic acid and 0.08 M sodium acetate for 30 min. The positive signal was visualized as a dark brown color.

Real-time PCR

Total RNAs from mouse uteri or cultured cells were isolated using TRIPURE reagent according to the manufacturer's

instructions (Roche) and reverse transcribed into cDNA using M-MLV reverse transcriptase (Promega). Reverse transcription was performed at 42 °C for 60 min with 2 µg total RNA in 25 µl volume. For real-time PCR, cDNA was amplified using FS Universal SYBR Green Real Master (Roche) on BIO-RAD CFX96 Real Time Detection System. The conditions used for real-time PCR were as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All reactions were run in triplicate. The result was analyzed using CFX Manager Software. After analysis using the $2^{-\Delta\Delta Ct}$ method, data were normalized to *Gapdh* expression. Primer sequences for real-time PCR were as follows: *Cryab* (5'-CGGACTCTCAGATGCGT and 5'-TGGGATCCGGTACTTCCTGT), *Gapdh* (5'-GCCTTCCGTGTTCTACCC and 5'-TGCCTGCTTCACACCTTC), and *Prl8a2* (5'-AGCCAGAAATCACTGCCACT and 5'-TGATCCATGCACCCATAAAA).

Isolation of uterine stromal cells

Uterine stromal cells were isolated as previously reported (Liang *et al.* 2010). Briefly, uterine horns from day 4 pregnant mice were split longitudinally to expose the uterine lumen and digested in Hanks' balanced salt solution (HBSS) containing 1% trypsin (Amresco, Solon, OH, USA) and 6 mg/ml dispase (Roche). The digested uteri were shaken lightly to remove sheets of luminal epithelial cells. After the supernatant containing sheets of epithelial cells was discarded, the remaining tissues were washed three times with HBSS and incubated in HBSS containing 0.15 mg/ml collagenase I (Invitrogen) at 37 °C for 30 min, followed by vigorous shaking until the supernatant became turbid. The supernatant was then passed through a 70 µm wire gauze filter to eliminate epithelial sheets and centrifuged. The cell pellets were washed twice with HBSS and resuspended in complete medium consisting of DMEM-nutrient mixture F-12 Ham (DMEM-F12; Sigma) with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies, Inc.). Viable cells were counted by trypan blue staining using a hemocytometer. Cells were plated onto 35 mm culture dishes at the concentration of 1×10^6 cells/ml. After an initial culture for 30 min, the medium was changed to remove free-floating cells. The isolated stromal cells were further cultured in fresh complete medium at 37 °C with 5% CO₂ before treatments.

In vitro decidualization

In vitro decidualization of endometrial stromal cells from day 4 of pregnancy was performed as described previously (Liang *et al.* 2010). Briefly, primary uterine stromal cells isolated on day 4 pregnancy were induced for *in vitro* decidualization with fresh medium supplemented with progesterone (100 nM) and estrogen (0.1 nM) in DMEM-F12 with 2% charcoal-treated FBS (Biological Industries Ltd., Kibbutz Beit Hemeek, Israel).

Steroid hormonal treatments in vitro

Cultured stromal cells were treated with 100 nM progesterone or 0.1 nM estrogen. For further studies, cells were pretreated with RU486 (1 µM) or ICI 182 780 (100 nM) before the

addition of progesterone or estrogen respectively. Then cells were collected at 24 h for further quantitative analysis by real-time PCR. All steroids and antagonists were dissolved in ethanol. Controls received the vehicle only.

Statistical analysis

All the experiments were independently repeated at least three times. The significance of differences was analyzed by one-way ANOVA using the SPSS software program (SPSS, Inc.). The differences were considered significant at $P < 0.05$.

Declaration of interest

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

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