RESEARCH

Progesterone-regulated Hsd11b2 as a barrier to balance mouse uterine corticosterone

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Abstract

Glucocorticoids (GCs) are essential for mouse embryo implantation and decidualization. Excess GCs are harmful for mouse embryo implantation and decidualization. 11 β -Hydroxysteroid dehydrogenases type I and II (Hsd11b1/Hsd11b2) are main enzymes for regulating local level of GCs. Hsd11b2 acts as the placental glucocorticoid barrier to protect the fetus from excessive exposure. Although effects of GCs on the fetus and placenta in late pregnancy have been extensively studied, the effects of these adrenal corticosteroids in early pregnancy are far less well defined. Therefore, we examined the expression, regulation and function of Hsd11b1/Hsd11b2 in mouse uterus during early pregnancy. We found that Hsd11b2 is highly expressed in endometrial stromal cells on days 3 and 4 of pregnancy and mainly upregulated by progesterone (P4). In both ovariectomized mice and cultured stromal cells, P4 significantly stimulates Hsd11b2 expression. P4 stimulation of Hsd11b2 is mainly mediated by the Ihh pathway. The uterine level of corticosterone (Cort) is regulated by Hsd11b2 during preimplantation. Embryo development and the number of inner cell mass cells are suppressed by Cort treatment. These results indicate that P4 should provide a low Cort environment for the development of preimplantation mouse embryos by promoting the expression of uterine Hsd11b2.

Key Words

- ▶ progesterone
- ▶ corticosterone
- ▶ Hsd11b2
- ▶ endometrium
- ▶ glucocorticoid barrier

Journal of Endocrinology
(2020) **244**, 177–187

Introduction

Uterine receptivity and decidualization are critical events for the success of pregnancy in mice and humans (Dey *et al.* 2004, Cha *et al.* 2012). P4 is necessary for the establishment and maintenance of pregnancy in almost all mammals (Tu *et al.* 2014). P4 acts mainly through intracellular progesterone receptor (PGR). PGR-deficient mice are infertile (Lydon *et al.* 1995, Bhurke *et al.* 2016).

Ihh, a member of the hedgehog gene family, is a secretory protein regulated by P4 and expressed in the uterine epithelium during preimplantation (Bhurke *et al.* 2016). Epithelial Ihh triggers the recruitment and activation of the intracellular transducer Smoothened

by binding to its receptor Patched (Ptc) in stromal cells (McMahon 2000). These signaling events activate the downstream transcription factors Gli1-3 and chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII), which regulate cell proliferation and differentiation (Bhurke *et al.* 2016). Uterine epithelium-specific deletion of PGR shows reduced expression of Ihh with loss of epithelial PGR (Franco *et al.* 2012). Uterine-specific deletion of Ihh leads to infertility due to a defect in embryo implantation (Lee *et al.* 2006). Further studies show that loss of epithelial Ihh is also associated with aberrant gene expression in the stroma, suggesting that Ihh regulates stromal function via paracrine mechanisms

(Kurihara *et al.* 2007, Bhurke *et al.* 2016). These results indicate that the PGR-Ihh signaling pathway mediates epithelial-stromal interaction in the endometrium.

GCs, steroid hormones released by the adrenal cortex, are involved in the establishment and maintenance of pregnancy in mice (Whirledge *et al.* 2015). However, accumulating evidence suggests that exposure to high levels of GCs during pregnancy is detrimental for both embryo implantation and fetal development (Seckl & Meaney 2004, Zhao *et al.* 2013, Jafari *et al.* 2017, Li *et al.* 2018). Hsd11b1/Hsd11b2 are main enzymes for regulating local level of GCs (cortisol in humans; corticosterone in mice). Hsd11b1 catalyzes the conversion of inactive cortisone and 11-dehydrocorticosterone into active cortisol and corticosterone, respectively. In contrast, Hsd11b2 catalyzes the conversion of active cortisol and corticosterone into inactive cortisone and 11-dehydrocorticosterone, respectively (Sandeep & Walker 2001). Hsd11b2, as the placental GC barrier, is mainly located in the syncytial layer of the placental villi to protect the fetus from excessive cortisol or corticosterone exposure (Togher *et al.* 2014, Zhu *et al.* 2019). Fetal GC levels are lower than maternal levels (Beitins *et al.* 1973, Seckl & Walker 2001). Reduced placental Hsd11b2 activity and mRNA are observed in a subgroup of fetuses with intrauterine growth restriction (Dy *et al.* 2008). Both chronic restraint stress and feed restriction cause an increase in maternal Cort levels and reduced placental Hsd11b2 mRNA expression, which result in lower fetal birth weight in pregnant rats (Togher *et al.* 2014). This evidence indicates that placental Hsd11b2 plays a major role during pregnancy when the fetus is exposed to maternal GCs. However, whether there is a uterine GC barrier before placentation is largely unknown. GCs are used to treat infertile patients with repeated IVF failure and recurrent miscarriage (Boomsma *et al.* 2012). It seems that the fertility-promoting or -inhibiting effects of GCs are dependent on timing, dose, and responsiveness within a given tissue (Robertson *et al.* 2016). Although effects of GCs on the fetus and placenta in late pregnancy have been extensively studied, the effects of these adrenal corticosteroids in early pregnancy are far less well defined.

To address this issue, we examined the spatiotemporal expression and function of Hsd11b2 during early pregnancy. Our data showed that uterine Hsd11b2 plays an important role in maintaining GC balance during preimplantation.

Materials and methods

Animals and treatments

All animal experiments were approved by the Animal Care and Use Committee of South China Agricultural University. All CD1 mice were housed in a controlled environment with a 14 h light:10 h dark cycle. Female mice, aged 8–10 weeks, were mated with fertile or vasectomized males to induce pregnancy or pseudopregnancy (day 1 is the day of vaginal plug). From days 1 to 4, pregnancy was confirmed by flushing the embryos from the oviducts or uteri.

Ovariectomized mice were used to examine the effects of P4 following 2 weeks of recovery. The ovariectomized mice received a single s.c. injection of P4 (1 mg/mouse, Sigma-Aldrich). The control mice received vehicle (sesame oil, 0.1 mL/mouse) only. Mice were killed to collect uteri at different time points after P4 injections. RU486, an antagonist of PGR, was subcutaneously injected (8 mg/kg body weight, Cayman Chemical) to examine PGR involvement of P4 regulation (Cheon *et al.* 2002). The control mice received vehicle (sesame oil, 0.1 mL/mouse) only. Mice were killed 24 h later to collect uteri.

To examine the effects of P4 on Cort level in uterine lumen fluid, ovariectomized mice received a single s.c. injection of vehicle (sesame oil, 0.1 mL/mouse) or P4 (1 mg/mouse). After 12 h of injection, ovariectomized mice received a single s.c. injection of vehicle (DMSO, 0.1 mL/mouse) or Cort (1 mg/mouse, Cayman Chemical). Mice were killed 3 h later to collect the fluid from uterine lumen.

Single prolonged stress (SPS) tests were performed as previously described (Deslauriers *et al.* 2018). Briefly, ovariectomized mice underwent a restraint-immobilization stress test for 2 h and forced swimming for 20 min in 24°C water. They were allowed to recuperate for 15 min. Finally, the mice were exposed to diethyl ether until loss of consciousness. To compare with SPS, ovariectomized mice were intraperitoneally injected with Cort (1 mg/mouse in 0.1 mL DMSO). The control mice were intraperitoneally injected with 0.1 mL DMSO. Mouse serum was collected 1 h after SPS or Cort injection. Uterine segments were collected 6 h after SPS or Cort injection.

In situ hybridization

Total RNAs from mouse kidneys were reverse-transcribed, and each template of hybridization probe was amplified

Table 1 Primers used in this study.

Gene	ID	Application	Primers (5'–3')	Products (bp)
<i>Rpl7</i>	M29016	RT-PCR	GCAGATGTACCGCACTGAGATTC ACCTTTGGGCTTACTCCATTGATA	129
<i>Hsd11b1</i>	NM_008288.2	ISH	GCTGGCACTATGGAAGAC CCTTGGTTATGTAGAGTTCTG	349
<i>Hsd11b2</i>	NM_008289.2	ISH	TGTGAACCTCTGGGAGAAACG CGGGGCAGAAGGTGATTG	306
<i>Hsd11b1</i>	NM_008288.2	RT-PCR	CGACATCCACTCTGTGCGAA TGCTGCCATTGCTCTGCT	101
<i>Hsd11b2</i>	NM_008289.2	RT-PCR	TGTGAACCTCTGGGAGAAACG GCATCTACAACTGGGCTAAGGT	156
<i>lhh</i>	NM_001313683.1	RT-PCR	CTGCGGTTCTGTCTGTTT GTTCTCCTCGTCCTTGAAG	261
<i>Dhh</i>	NM_007857.5	RT-PCR	GACCGTGACCGTAATAAGTA GACCGCCAGTGAGTTATC	126
<i>Shh</i>	NM_009170.3	RT-PCR	CAGCGGCAGATATGAAGG GAGACTCCTCTGAATGATGG	242
<i>Gli1</i>	NM_010296.2	RT-PCR	TTCATCAACTCTCGCTGTA TTGCCAACCATCATATCCA	242
<i>Gli2</i>	NM_001081125.1	RT-PCR	GTCACATCAGCCAACCAA AGCCTCCATTCTGTTCTATAC	163
<i>Gli3</i>	NM_008130.3	RT-PCR	CTGCGGTATCTCTCTCA TAGTGCTGGTATTGCTGTC	156

with the corresponding primers (Table 1). pGEM-T vector plasmid (Promega) was used to clone the amplified fragments of different genes. Digoxigenin-labeled antisense or sense cRNA probes were transcribed *in vitro* by using a digoxigenin RNA labeling kit (Roche Diagnostics). *In situ* hybridization was performed as previously described (Lei *et al.* 2012, Ding *et al.* 2018).

Real-time RT-PCR

Total RNAs from mouse uteri or cultured cells were isolated using TRIzol reagent (TaKaRa, Tokyo, Japan), digested with RQ1 deoxyribonuclease I (Promega) and reverse transcribed into cDNA with a PrimeScript reverse transcriptase reagent kit (Vazyme, Nanjing, Jiangsu, China). cDNA was amplified using a SYBR Premix Ex Taq kit (Vazyme) on the BIORAD-CFX96™ Real-Time System (Bio-Rad) according to the manufacturer's recommendations. The specificity of each pair of primers for each gene was checked through the melting curve to make sure a single peak. All reactions were run in triplicate. All primer sequences used for real-time RT-PCR were listed in Table 1. The real-time values are normalized to the RPL7 expression level and indicated as the mean $\pm$ S.E.M.

Western blot

The uterine tissues were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.5, 0.1% SDS, 150 mM NaCl,

1% Triton X-100, and 0.25% sodium deoxycholate) and supplemented with protease inhibitor cocktail (Roche) on ice. Cultured cells were scratched directly in lysis buffer. Protein concentration was measured with a bicinchoninic acid assay reagent kit (Appligen, Beijing, China). Protein samples were separated by SDS-PAGE and transferred onto PVDF membranes (Merck Millipore). After blocking in 5% nonfat powdered milk (Tris-buffered saline containing 0.1% Tween-20) for 1 h, membranes were incubated with each primary antibody overnight at 4°C as follows: anti-Hsd11b2 (Santa Cruz Biotechnology) and anti- α -Tubulin (Cell Signaling Technology). Western blot was performed as previously described (Lei *et al.* 2012).

Isolation and culture of endometrial stromal cells

Mouse endometrial stromal cells were isolated as previously described (Lei *et al.* 2012). Briefly, uterine horns from day 4 pregnant mice were split longitudinally and digested in Hanks' balanced salt solution containing 1% trypsin (Ameresco, Solon, OH, USA) and 6 mg/mL dispase II (Roche). After removing luminal epithelial cells, the remaining uteri were incubated with 0.15 mg/mL collagenase I (Invitrogen). Primary endometrial stromal cells were plated onto culture plates. Cultured stromal cells were treated with P4, RU486, Cyclopamine (TargetMol, Shanghai, China), Purmorphamine (Selleck Chemicals), GANT61 (TargetMol) or Cort, respectively. The doses of reagents used for treating stromal cells were chosen based

on previous publications (Wu *et al.* 2004, Mazumdar *et al.* 2011, Lei *et al.* 2012). Then cells were collected at different time points for further quantitative analysis by real-time RT-PCR.

Explant culture

The uterine explant culture was performed as previously described (Matsumoto *et al.* 2002). Briefly, uteri from day 4 pregnant mice were slit longitudinally from the mesometrial side and digested in Hanks' balanced salt solution containing 1% trypsin. After removing luminal epithelial cells, the remaining uteri were cut into 1.5–2 mm long pieces. Individual pieces were incubated in DMEM containing 10% FBS for 3 h. After 3 h of incubation, uterine explants were placed in fresh medium with or without Ihh (2 µg/mL; R&D Systems) and cultured for 48 h. Uterine explants were collected for real-time RT-PCR analysis.

Measurement of Cort level in uterine lumen fluid

Concentrations of Cort were measured by Cort ELISA Kit (Cayman Chemical, Ann Arbor, MI) per manufacturer instructions. Uterine lumen fluid was collected directly by intraluminal infusion of 200 µL of PBS. The uterine fluid was mixed with four-fold volume of dichloromethane and vortexed for 2 min. The dichloromethane layer (lower layer) was transferred into a clean test tube with a liquid shifter. Cort in the samples was extracted with dichloromethane three times. After evaporating the methylene chloride under a gentle stream of nitrogen, the extracts were dissolved in ELISA Buffer and measured by the ELISA Kit.

Embryo culture and immunofluorescence

All two-cell embryos were obtained by flushing mouse oviducts in the morning on day 2 of pregnancy and washed four times in M2 medium (Sigma-Aldrich). All embryos were cultured in KSOM-AA medium (Merck Millipore) with or without Cort. Cort was dissolved in DMSO and diluted in KSOM-AA medium. The final concentrations of Cort were 0.1, 1 and 10 µM. The rates of blastocyst formation and hatching were examined. Embryos were collected for immunofluorescence at the blastocyst stage. Blastocysts were fixed in 4% paraformaldehyde for 1 h, washed three times in washing buffer (PBS containing 0.1% Tween-20) and permeabilized with PBS containing 0.5% Triton X-100 for 15 min. Embryos were blocked with 5% donkey

serum for 1 h at 37°C and incubated overnight with rabbit anti-mouse Oct4 (Abcam) at the appropriate dilutions at 4°C. After washing in washing buffer, embryos were incubated with an anti-rabbit antibody-conjugated with Alexa Fluor 488 (Jackson ImmunoResearch). Then nuclei were stained with propidium iodide (Sigma-Aldrich). The fluorescence was visualized under Leica Laser Scanning Confocal Microscopy.

Statistical analysis

All the experiments were independently repeated at least three times. Data were processed using GraphPad Prism 6 Software and reported as the mean ± S.E.M. Student's *t*-test was used for the comparison between two groups. For multiple comparisons, ANOVA with *post hoc* Tukey was used. Statistical significance is indicated by **P* < 0.05.

Results

Hsd11b1/2 expression in mouse uteri during early pregnancy

The local inter-conversion between inactive 11-dehydrocorticosterone and active Cort is regulated by Hsd11b1/2 in mice. To explore whether the local conversion was present in the mouse uterus during early pregnancy, *in situ* hybridization was performed to examine the localization of *Hsd11b1/2* mRNA.

Our results showed no detectable *Hsd11b1* signal from days 1 to 7 of pregnancy. A strong *Hsd11b1* signal was detected in the secondary decidual zone on day 8 of pregnancy (Fig. 1A). *Hsd11b2* signals were mainly seen in endometrial stromal cells from days 3 to 4 of pregnancy (Fig. 1A). On day 5, *Hsd11b2* was weakly expressed in the primary decidual cells at implantation sites (Fig. 1A).

Data from real-time PCR also showed that *Hsd11b2* mRNA levels were upregulated significantly in uteri on days 3 and 4 compared to days 1 and 2 (Fig. 1B). The protein levels of Hsd11b2 on days 3 and 4 were also significantly higher than that on days 1 and 2 (Fig. 1C). Because *Hsd11b1* expression was very low during preimplantation, we mainly focused on *Hsd11b2*.

P4 regulation on Hsd11b2 expression in mouse uterus

Our data showed that expression of *Hsd11b2* was highest on days 3 and 4 during early pregnancy (Fig. 1). Moreover, P4 level also increased significantly during this period

Figure 1

Spatiotemporal expression of Hsd11b1 and Hsd11b2 in mouse uteri during early pregnancy. (A) *In situ* hybridization of *Hsd11b1* and *Hsd11b2* mRNA expression in mouse uteri on days 1–8 of pregnancy. *Hsd11b1* was mainly localized in secondary decidual zone on day 8, whereas *Hsd11b2* was highly expressed in stromal cells on days 3–4. s, stroma; le, luminal epithelium; e, embryo. Scale bar, 200 μ m. (B) Real-time RT-PCR analysis of uterine *Hsd11b2* mRNA levels on days 1 ($n = 9$), 2 ($n = 9$), 3 ($n = 9$) and 4 ($n = 9$) of pregnancy. (C) Western blot analysis of uterine Hsd11b2 protein levels on days 1 ($n = 6$), 2 ($n = 6$), 3 ($n = 6$) and 4 ($n = 6$) of pregnancy. $*P < 0.05$. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0349>.

(Wang & Dey 2006). Therefore, we explored whether there was a strong link between P4 and Hsd11b2. Treatment of ovariectomized mice with P4 for 12 h or 24 h caused a significant increase of Hsd11b2 mRNA and protein levels (Fig. 2A and B). To investigate whether P4 regulated the expression of Hsd11b2 through PGR, mice on day 3 of pregnancy were treated with RU486, an antagonist of PGR. Uterine *Hsd11b2* mRNA levels were obviously down-regulated by RU486 for 24 h (Fig. 2C). To further evaluate the effects of P4, uterine stromal cells isolated from day 4 of pregnancy were treated with P4 or/and RU486 for 24 h. *Hsd11b2* expression was significantly induced by P4, which was obviously abrogated by RU486 (Fig. 2D). These results indicated that P4 regulated Hsd11b2 expression in a PGR-dependent manner.

Regulation of Hsd11b2 by Ihh pathway of epithelial-stromal interaction

Ihh, a target gene of P4, is highly expressed in the luminal and glandular epithelium on days 3 and 4 of gestation (Paria *et al.* 2001, Matsumoto *et al.* 2002, Takamoto *et al.* 2002, Lee *et al.* 2006). *Ptc*, the *Ihh* receptor, is also observed in uterine stromal cells on days 4–5 of pregnancy (Matsumoto *et al.* 2002), suggesting that *Ihh*, a diffusible factor from the epithelium, regulates stromal functions by paracrine mechanisms. We assumed that *Ihh* may regulate Hsd11b2 in the uterus. We found that *Ihh* was induced by P4 in ovariectomized mice (Fig. 3A). Since Sonic HH (Shh) and Desert HH (Dhh) in HH family also activate downstream hh signaling pathways,

Figure 2

Effects of P4 on Hsd11b2 expression. (A) Uterine *Hsd11b2* mRNA levels after ovariectomized mice received a single injection of oil or P4 for 12 h and 24 h, respectively. $n = 12$. (B) Western blot analysis of uterine Hsd11b2 protein levels 24 h after ovariectomized mice received a single injection of P4 (1 mg/mouse). $n = 12$. (C) Real-time RT-PCR analysis of uterine *Hsd11b2* mRNA levels after mice on day 3 were treated with RU486 for 24 h. $n = 6$. (D) Real-time RT-PCR analysis of *Hsd11b2* mRNA levels after stromal cells were treated with 1 μ M P4 or/and 1 μ M RU486 for 24 h. Ctrl, Control; P4, progesterone; RU486, a PGR antagonist. * $P < 0.05$.

we examined whether they were regulated by progesterone. Our results showed that *Shh* expression was not detected by real-time RT-PCR, and *Dhh* expression remained unchanged by P4 treatment for 6 h (Fig. 3A). To study whether the *Ihh* signaling pathway was involved in the expression of *hsd11b2*, the uterine explants were treated with recombinant *Ihh*. The expression of *Hsd11b2* was significantly induced by *Ihh* (Fig. 3B). *Hsd11b2* expression in stromal cells was obviously suppressed by Cyclopamine, a specific antagonist of Smoothened in *Ihh* pathway (Fig. 3C). Furthermore, stromal *Hsd11b2* expression was significantly stimulated by purmorphamine, an agonist of Smoothened (Fig. 3D and E). These results indicated that the expression of *Hsd11b2* was regulated by *Ihh* signaling.

Although mRNA levels of *Gli1*, *Gli2* and *Gli3* increased during preimplantation period, the levels of *Gli1* were much higher than *Gli2* and *Gli3*, especially on days 3 and 4 (Fig. 4A). Furthermore, only *Gli1* was induced by *Ihh* and Purmorphamine *in vitro* (Fig. 4B and C). The expression of *Hsd11b2* was obviously downregulated after stromal cells were treated with GANT61, an inhibition of

Figure 3

Hsd11b2 regulation by P4 through the *Ihh* pathway. (A) Uterine *Ihh* and *Dhh* mRNA levels after ovariectomized mice received a single injection of oil or 1 mg/mouse P4 for 6 h. $n = 6$. (B) The effect of recombinant *Ihh* on uterine *Hsd11b2* mRNA levels in explant cultures. Longitudinally split uterine pieces devoid of luminal epithelium were cultured for 48 h in the absence or presence of *Ihh* (2 μ g/mL). $n = 6$. (C) Real-time RT-PCR analysis of *Hsd11b2* mRNA levels after stromal cells were treated with different doses of Cyclopamine for 24 h. Cyclopamine, a Smoothened antagonist in *Ihh* pathway. (D) Real-time RT-PCR analysis of *Hsd11b2* mRNA after stromal cells were treated with different doses of Pur for 24 h. (E) Western blot analysis of Hsd11b2 protein after stromal cells were treated with different doses of Pur for 24 h. Pur, Purmorphamine, a Smoothened agonist. * $P < 0.05$.

Ihh pathway for *Gli1/2* (Fig. 4D). Therefore, *Gli1* was the dominant form for *Ihh* regulation of *Hsd11b2*.

Effects of P4 on uterine Cort

To further analyze the role of *Hsd11b2* on uterine GC balance, we compared Cort levels in uterine lumen fluid between days 2 and 3 of pregnancy. Cort levels on day 3 were significantly lower than those on day 2 (Fig. 5A). Considering P4 upregulation on *Hsd11b2*, we anticipated that P4 may have the ability to downregulate Cort level

Figure 4

Expression levels of *Gli1*, *Gli2* and *Gli3*. (A) Real-time RT-PCR analysis of *Gli1*, *Gli2* and *Gli3* mRNA levels on days 1–4 of pregnancy. (B) The effect of recombinant Ihh on uterine *Gli1*, *Gli2* and *Gli3* mRNA levels in explant cultures. (C) Real-time RT-PCR analysis of *Hsd11b2* mRNA levels after stromal cells were treated with 2.5 μM Pur for 24 h. Pur, Purmorphamine. (D) Real-time RT-PCR analysis of *Hsd11b2* mRNA levels after stromal cells were treated with different doses of GANT61 for 24 h. GANT61, an inhibitor for *Gli1* and *Gli2*. **P* < 0.05.

in uterine fluid by promoting expression of *Hsd11b2*. To confirm, we injected exogenous Cort into ovariectomized mice and found that the concentration of Cort in the uterine lumen was nearly ten-fold higher than that in the control group. This increase of Cort was abrogated by pretreatment with P4 12 h before Cort injection (Fig. 5B). Meanwhile, we also examined Cort levels in serum. We observed that serum levels of Cort were significantly higher in the Cort-treated group than in the untreated group (Fig. 5C). However, the serum levels of Cort were not significantly different between the P4-pretreated group and the untreated group after ovariectomized mice received a single injection of Cort (Fig. 5C), suggesting that P4 only downregulated uterine Cort but had no effect on systemic Cort. Meanwhile, *Hsd11b2* expression was not only increased by P4 pretreatment, but also by treatment of both P4 and Cort (Fig. 5D). However, uterine *Hsd11b1* expression remained relatively stable after ovariectomized mice were treated by P4 or Cort (Fig. 5E).

Effects of Cort and SPS on uterine *Hsd11b2*

Because GCs are mainly involved in stress responses, we analyzed whether *Hsd11b2* expression was affected by SPS. Serum levels of Cort were significantly increased

Figure 5

Effects of P4 on uterine Cort concentration. (A) The levels of Cort in the fluid of uterine lumen on days 2 (*n* = 10) and 3 (*n* = 9). (B) Cort levels in uterine fluid after ovariectomized mice received a single injection of P4 or Cort. *n* = 24. (C) Serum levels of Cort after ovariectomized mice received a single injection of P4 or Cort. (D) Uterine *Hsd11b2* mRNA level after ovariectomized mice received a single injection of P4 or Cort. (E) Uterine *Hsd11b1* mRNA level after ovariectomized mice received a single injection of P4 or Cort. Cort, corticosterone.

1 h after ovariectomized mice underwent SPS (Fig. 6A). In order to verify that the effect of SPS on *Hsd11b2* is mainly played by Cort, a single injection of exogenous Cort (1 mg/mouse) of ovariectomized mice also caused a sharp increase of serum Cort level in 1 h (Fig. 6B). Uterine *Hsd11b2* expression was significantly increased 6 h after SPS (Fig. 6C). Injection of exogenous Cort also stimulated uterine *Hsd11b2* mRNA expression (Fig. 6D). Moreover, *Hsd11b2* mRNA levels were significantly increased when cultured stromal cells were treated with 1 μM or 10 μM Cort for 24 h (Fig. 6E).

Effects of Cort on embryo development in vitro

Our data showed that exogenous injection of Cort could induce an increase of Cort levels in the

Figure 6

Effects of SPS and Cort on Cort level. (A) The serum levels of Cort 1 h after ovariectomized mice underwent SPS. $n = 9$. (B) The serum levels of Cort 1 h after ovariectomized mice received a single injection of exogenous Cort. $n = 9$. (C) Uterine *Hsd11b2* mRNA levels 6 h after ovariectomized mice received a SPS. $n = 9$. (D) Uterine *Hsd11b2* mRNA levels 6 h after ovariectomized mice received a single injection of exogenous Cort for 6 h. $n = 9$. (E) The *Hsd11b2* mRNA levels after stromal cells were treated with different concentrations of Cort for 24 h.

uterine lumen (Fig. 5B). Therefore, we evaluated the effect of Cort on the development of mouse embryos *in vitro*. After two-cell mouse embryos were treated with different concentrations of Cort, 100 μ M Cort reduced the rate of blastocyst formation, but 1 or 10 μ M Cort had no obvious effects (Fig. 7A). Only 100 μ M Cort significantly reduced the rate of embryo hatching (Fig. 7B). To further analyze the potential effect of Cort on embryo development, we counted the number of inner cell mass cells shown by immunofluorescence with anti-Oct4 antibody. Our analysis showed that the number of inner cell mass cells significantly decreased after two-cell embryos were cultured with 0.1, 1 or 10 μ M Cort compared to the control group (Fig. 7C).

Discussion

In our study, Hsd11b1 is mainly localized in the endometrial secondary decidual zone on day 8 of pregnancy. A previous study reported that Hsd11b1 mRNA and protein levels progressively increased from day 5 to the last day of pregnancy in mouse uteri (Damiani *et al.* 2017). Data from uterine GR KO mice indicates that the GR-mediated GC signaling pathway is necessary for embryo implantation and decidualization (Whirledge *et al.* 2015). In the mouse, GR stimulates the induction of WNT4, a key regulator of progesterone signaling during decidualization (Franco *et al.* 2011). Indeed, HSD11B1 is expressed at its highest levels in the human endometrium decidua (Michael & Papageorgiou 2008). *In vitro* decidualization in humans induces strong *HSD11B1* expression (105-fold) (Takano *et al.* 2007). These data suggest that local Cort generated by Hsd11b1 likely plays an important role for the process of decidualization.

In our study, Hsd11b2 mRNA and protein levels have an obvious increase from day 3 to 4 of pregnancy. Because the Hsd11b2 expression pattern is matched with a high level of P4 during preimplantation (Egashira & Hirota 2013), we further showed that Hsd11b2 expression is regulated by Ihh signaling. P4 stimulates stromal Hsd11b2 expression through secreted Ihh from the luminal epithelium via Smoothened and Gli1. Ihh is strongly expressed in the uterine epithelium of the preimplantation mouse uterus and regulated by progesterone (Matsumoto *et al.* 2002, Takamoto *et al.* 2002). Embryo implantation fails in mice with uterus-specific Ihh deletion (Lee *et al.* 2006). The loss of epithelial Ihh also results in aberrant gene expression in the stroma (Kurihara *et al.* 2007, Bhurke *et al.* 2016).

In the Ihh pathway, downstream transcription factors Gli1 and Gli2 of Smoothened are upregulated from day 3 of pregnancy (Matsumoto *et al.* 2002). In both human primary cytotrophoblasts and trophoblast-like BeWo cells, HSD11B2 expression is regulated by SHH/GLI2 signaling (Zhu *et al.* 2016). In our study, Shh expression in the mouse uterus during preimplantation is under the detectable range by real-time RT-PCR and is not regulated by P4. Furthermore, P4 has no effect on Dhh expression, which is expressed at a low level. Our data suggest that Ihh is the dominant form in mediating Hsd11b2 expression.

Although the abundant expression of placental Hsd11b2 has been believed to function mainly as a barrier for maternal GCs (Zhu *et al.* 2019), the expression and function of Hsd11b2 have not been reported during early pregnancy in mice. Recent data showed that high

Figure 7

Effects of Cort on the development of mouse embryos *in vitro*. (A) The rate of blastocyst formation after two-cell embryos were cultured with 0 ($n = 78$), 1 ($n = 98$), 10 ($n = 86$) or 100 μM ($n = 90$) Cort to blastocyst stage. (B) The rate of blastocyst hatching after two-cell embryos were cultured with different concentrations of Cort to blastocyst stage. (C) The number of inner cell mass expressing Oct4 after two-cell embryos were cultured with 0 ($n = 61$), 0.1 ($n = 56$), 1 ($n = 51$) or 10 μM ($n = 43$) of Cort to blastocyst stage. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0349>.

levels of Cort are detrimental for mouse receptivity and decidualization (Zhao *et al.* 2013, Li *et al.* 2018). The high levels of endometrial Hsd11b2 before implantation suggest that the uterus provides an environment of low-level Cort for embryo implantation. Isolated endometrial stromal cells and embryo are exposed to physiological concentrations (1×10^{-7} M, Gong *et al.* 2015) and supra-concentrations of Cort (1×10^{-6} M and 1×10^{-5} M). The results show that supra-concentrations Cort promote the expression of *Hsd11b2* mRNA levels in stromal cells. This suggests that endometrial stromal cells provide a potential for reducing high concentrations of Cort by Hsd11b2. Based on our data and previous studies by other groups, mouse embryos are resistant to high levels of Cort, considering the rate of blastocyst formation and hatching (Zhao *et al.* 2013, Li *et al.* 2018). However, inner cell mass development is sensitive to Cort exposure, unlike blastocyst formation and hatching, as the number of inner cell mass cells is significantly reduced by a lower concentration of Cort.

Because mouse preimplantation embryos are sensitive to Cort, the high level of Hsd11b2 in the uterus on

days 2 and 3 provides a low-level Cort environment for preimplantation development of mouse embryos. Our studies showed that the concentration of Cort in the uterine lumen fluid is significantly reduced by P4-induced Hsd11b2 expression. Additionally, the stimulated increase of Hsd11b2 levels by SPS or Cort injection triggers protective mechanisms to prevent damage to embryos by high levels of maternal Cort. The high expression of placental Hsd11b2 acts as a GC barrier to protect the fetus from excessive maternal Cort exposure (Seckl & Walker 2001, Zhu *et al.* 2019). It is possible that endometrial Hsd11b2 regulated by P4 provides a suitable GC environment in the uterine lumen for preimplantation development. HSD11B2 is expressed in normal endometria during the menstrual cycle at higher levels than HSD11B1 (McDonald *et al.* 2006), suggesting the same mechanism may also work in human reproduction.

In conclusion, our data showed that high levels of uterine Hsd11b2 expression during preimplantation is regulated by P4 via *Ihh* signaling pathway. The strong expression of Hsd11b2 should provide a suitable Cort environment during preimplantation for mouse embryos.

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.

Funding

This work was supported by National Key Research and Development Program of China (2018YFC1004403) and National Natural Science Foundation of China (31471397, 31272263 and 31671563).

Author contribution statement

H T Z and Z M Y designed experiments; T F, Z S Y, and H T Z performed research and acquired data; T F, H Y Z, and H T Z analyzed data; T H Z, Z S Y, and Z M Y interpreted data; H T Z, H Y Z and Z M Y wrote the paper and revised it; all authors gave the final approve of the version to be published.

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Received in final form 7 October 2019

Accepted 10 October 2019

Accepted Manuscript published online 10 October 2019