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Oncostatin M expression in the mouse uterus during early pregnancy promotes embryo implantation and decidualization

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Oncostatin M (OSM) is a member of the interleukin-6 (IL-6) family, which functions in embryo implantation and decidualization. The expression, function and regulation of Osm in mouse uteri during early pregnancy remain unknown. We show that *Osm* is mainly expressed in the uterine epithelium from days 1 to 4 of pregnancy and in decidual cells on day 5 of pregnancy. Osm promotes the attachment of Osm-soaked blue beads, which mimic blastocysts, to a pseudopregnant mouse uterus. Prostaglandin E2 (PGE_2)-induced Osm in mouse uterine epithelial cells upregulates the levels of Il-33 expression and phosphorylates Stat3. *In vitro* decidualization is significantly promoted by Osm. Our results indicate that PGE_2-induced Osm may mediate embryo implantation through Il-33 and participate in decidualization *via* the Stat3-Egr1 pathway.

Keywords: adhesion; decidualization; implantation; Osm; polyploidization

The synchronized interaction of the functional blastocysts with a receptive endometrium is essential for successful implantation. Embryo implantation is coordinated by ovarian hormones, transcription factors, growth factors, and cytokines [1]. In rodents and humans, the implanting blastocysts will invade the endometrium and uterine stromal cells and will undergo decidualization [2]. Meanwhile, the decidual cells appear polyploidy [3]. However, the underlying mechanisms during embryo implantation and decidualization are still not fully understood.

Oncostatin M (OSM) is a member of interleukin-6 (IL-6) family. IL-6 family consists of IL-6, IL-11, IL-27, IL-31, OSM, leukemia inhibitory factor (LIF), ciliary neurotrophic factor, cardiotrophin 1 and cardiotrophin-like cytokine factor 1 [4]. The IL-6 family have a common signal transducer glycoprotein 130 (gp130) and can activate JAK/STAT pathway, mainly the phosphorylation of Stat3 [5]. The OSM functions through two receptors: type 1 is a heterodimer of gp130 and LIF receptor, and type 2 is a heterodimer of gp130 and OSM receptor [6]. In mice, OSM acts mainly through OSMR and gp130 complex and shows a low affinity with LIFR [7,8]. Among IL-6 family, LIF-deficient mice show implantation failure and decidualization deficiency [9]. The female mice lacking in IL-11 receptor are infertile due to defective decidualization [10]. As a member of IL-6 family, OSM is expressed in decidual stromal cells in pregnant women and may play an important role in placental functions by stimulating the releasing of human chorionic gonadotrophin [11]. In mice, OSM is mainly found in the luminal epithelia surrounding embryos with a few stromal cells on day 5 during early pregnancy, but is absent in LIF-null mice [12]. After LIF signaling is inhibited, OSM expression is reduced in the stroma on day 6 of pregnancy [13]. These studies suggest a possible role of OSM during early pregnancy. However, the

Abbreviations
gp130, glycoprotein 130; HBSS, Hanks' balanced salt solution; HRP, horseradish peroxidase; IL-6, interleukin-6; IS, implantation site; LIF, leukemia inhibitory factor; OSM, oncostatin M; PGE_2, prostaglandin E2; S3I, Stat3 inhibitor; siRNA, small interfering RNA.

expression, regulation and function of OSM during mouse early pregnancy are still unknown.

In our study, we examined Osm expression and function in mouse uteri during early pregnancy. Osm is expressed at implantation sites (ISs) and may promote embryo implantation through Il-33. Osm also plays a role in decidualization *via* Egr1. Our study for the first time shows that Osm can promote the implantation and decidualization of the early pregnant mouse.

Materials and methods

Animals and treatments

All of our animal experiments were approved by the Institutional Animal Care and Use Committee of South China Agricultural University and executed in line with the institutional and national guidelines. Mature mice (CD1 strain) were maintained in a temperature- and light-controlled environment (12 h light and 12 h dark cycle). Mature female mice (8–10 weeks) were mated with fertile or vasectomized males of the same strain to induce pregnancy or pseudopregnancy (days 1 is the day of vaginal plug). From days 1–4, pregnancy was confirmed by recovering embryos from oviducts or uteri. As for days 5 and 6 of pregnancy, the ISs were verified by tail intravenous injection of 0.1 mL of 1% Chicago blue solution (1% in saline; Sigma, St. Louis, MO, USA).

In situ hybridization

Total RNAs from mouse uteri on days 5 of pregnancy were reverse transcribed and the template of each probe was amplified with the specific primers for mouse *Osm* and *Osmr* (Table 1). The amplified fragment of Osm and Osmr were cloned into pGEM-T plasmid (Promega, Madison, WI, USA), and verified by sequencing. Primers for T7 and SP6 were used to amplify Osm and Osmr template for labeling antisense probe of Osm and Osmr from the pGEM-Osm and Osmr plasmids. Digoxigenin-labeled antisense or sense cRNA probes were transcribed *in vitro* using a digoxigenin RNA labeling kit (Roche Applied Science, Indianapolis, IN, USA).

In situ hybridization was performed as previously described [14]. In brief, uteri were cut into 10 mm frozen sections on 3-aminopropyltriethoxy silane (Sigma)-treated slides and fixed in 4% paraformaldehyde solution in PBS. Hybridization was performed at 55 °C overnight. Digoxigenin-labeled Osm sense probe was used to replace OSM antisense probe for negative control. Following hybridization and posthybridization washing, sections were incubated in sheep anti-digoxigenin antibody conjugated with alkaline phosphatase at 4 °C overnight (1 : 5000; Roche). The signal was visualized by incubating with nitro blue tetrazolium (0.4 mM) and 5-bromo-4-chloro-3-indolyl phosphate (0.4 mM). Endogenous alkaline phosphatase activity was inhibited by levamisole (2 mM; Sigma). All of the sections were counterstained with 1% methyl green which was extracted by chloroform. The positive signal was visualized as a dark brown color.

Immunohistochemistry

Immunohistochemistry was conducted as described previously [15]. In brief, paraffin sections were deparaffinized and rehydrated. Antigen recovery was performed in 0.01 M sodium citrate buffer (pH 6.0) by microwaving for 10 min. Endogenous horseradish peroxidase (HRP) activity was treated with 3% H_2O_2 for 15 min. After blocking with 10% horse serum for 1 h at 37 °C, sections were incubated with goat anti-Osm (0.5 $\mu g \cdot mL^{-1}$; R&D systems, #AF-495-NA, Minneapolis, MN, USA) at 4 °C overnight. After washing, the sections were incubated with biotin-labeled rabbit anti-goat IgG antibody (Zhongshan Golden Bridge, Beijing, China) and streptavidin–HRP complex (Zhongshan Golden Bridge). According to the manufacturer's protocol, the positive signals were visualized using DAB HRP Color Development Kit (Zhongshan Golden Bridge). The nuclei were stained with hematoxylin.

Culture of two-cell embryos

Pregnant mice were sacrificed on day 2 to collect oviducts. Two-cell embryos were flushed from oviducts with M2 medium (Sigma, #M7167) on a 37 °C baking table. Following washing three times with M2 medium, two-cell embryos were cultured in KSOM (Merck, #MR-106-D, Darmstadt, Germany) containing different concentrations of Osm (Osm; R&D systems, #495-MO) for 60 h in 5% CO_2 incubator at 37 °C. The number of embryos that successfully developed to blastocyst stage in each group were recorded.

Transplantation of Osm-soaked blue beads

To determine the effect of Osm on embryo implantation, Affi-Gel blue Gel Beads (Bio-Rad, #1537302, Hercules, CA, USA) with a similar size with blastocyst were incubated with Osm (5 $ng \cdot \mu L^{-1}$) in 0.1% BSA/PBS at 37 °C for 2 h. After being washed with M2 (0.1% BSA) thrice, Osm-soaked beads (8 beads per horn) were transplanted into the uterine horns of day 4 pseudopregnant mice. Beads incubated in 0.1% BSA/PBS were transplanted as a control. Mice were sacrificed to observe ISs after beads transfer 48 h by tail intravenous injection of Chicago blue solution.

Embryo adhesion assay

The embryo adhesion assay was conducted as previously described [16]. In short, mouse uterine epithelial cells were

Table 1. Primer sequences used in our study

Gene	Primer sequences	Application	Accession number	Size (bp)
Osm	ATCCGCCTCCAAAACCTGAGCCATCGTCCCATTCCC	*In situ* hybridization	NM_001013365.2	433
Osmr	CAGATTATGACAGCGACTTCTTCCAGTAGCAGACAATGAT	*In situ* hybridization	NM_011019.3	349
Osm	GGCACAATATCCTCGGCATAAGTGTTGTAGTGGACCGTGAG	Real-Time PCR	NM_001013365.2	120
Osmr	GTAGACTGAACATATCCAACACGAGCCTTCTTCCAACACTTC	Real-Time PCR	NM_011019.3	271
Il-33	TGCATGAGACTCCGTTCTGGTCCCGTGGATAGGCAGAGAA	Real-Time PCR	NM_001164724.1	128
Egr1	GAGCGAACAACCCTATGAGGTCGTTTGGCTGGGATAA	Real-Time PCR	NM_007913	102
Dtprp	AGCCAGAAATCACTGCCACTTGATCCATGCACCCATAAAA	Real-Time PCR	NM_010088	119
Abp1	GGTGAGCGTGTTGCCTATGGAAAGCATCCAGGAAAGT	Real-Time PCR	NM_001161621	178
E2f8	GTGCTTCGTAGAACTCCCTGGCAATGTCATACAGCCTCCT	Real-Time PCR	NM_001013368.5	228
Rpl7	GCAGATGTACCGCACTGAGATTCACCTTTGGGCTTACTCCATTGATA	Real-Time PCR	M29016	129
siEgr1	CCTTCGCTCACTCCACTAT	siRNA transfection		
NC	CTCCGAACGTGTCACGT	siRNA transfection		

isolated and cultured in 24-well plates. After the epithelial cells were treated with 50 ng·mL^{-1} murine Il-33 (Peprotech, #210-33, Rocky Hill, NJ, USA) for 24 h, blastocysts collected from day 4 pregnant mouse uteri were transferred onto Il-33-treated epithelial cells and cultured further for 48 h. The epithelial cells treated with 0.1% BSA were served as a control. Then the numbers of the adhered blastocysts were counted and the adhesion rates were calculated.

Isolation and culture of uterine stromal cells

Mouse uterine stromal cells were isolated as described previously [17]. Uteri were washed with Hanks' balanced salt solution (HBSS; Sigma) digested with 1% (w/v) trypsin (Amresco, Cleveland, OH, USA) and 6 mg·mL^{-1} dispase (Roche Applied Science) in 3.5 mL HBSS for 1 h at 4 °C followed by 1 h at room temperature and 10 min at 37 °C. After the luminal epithelial cells were removed after HBSS washing, the remaining tissues were digested in 6 mL of HBSS containing 0.15 mg·mL^{-1} collagenase I (Invitrogen, 17100-017, Houston, TX, USA) at 37 °C for 35 min. The stromal cells were collected and cultured in DMEM/F12 (Sigma, # D2906) containing 10% heat-inactivated FBS (Biological Industries, Cromwell, CT, USA) after a 2-h initial culture in DMEM/F12 containing 2% heat-inactivated FBS to remove the unattached epithelial cells. In order to induce *in vitro* decidualization, endometrial stromal cells were treated with 10 nM estrogen (E2) and 1 μM progesterone (P4) in DMEM/F12 containing 2% charcoal-treated FBS (Biological Industries). For further analysis, stromal cells were treated with Osm (2, 10 and 50 ng·mL^{-1}) or Stat3 inhibitor (S3I, 100 μM; Merck, 573102).

Isolation and culture of uterine epithelial cells

Uterine epithelial cells were isolated as described previously [18]. Primary endometrial epithelial cells were enzymatically isolated from estrous female mouse uteri, judging by the redness of the vulva. Uteri were washed with HBSS (Sigma) and digested with 0.2% (w/v) trypsin (Amresco) and 6 mg·mL^{-1} dispase (Roche Applied Science) in 4.3 mL HBSS for 1.5 h at 4 °C followed by 30 min at room temperature. The luminal epithelial cells were collected after HBSS washing and cultured in DMEM/F12 containing 10% FBS. The epithelial cells were treated with Osm (10 and 50 ng·mL^{-1}), prostaglandin E2 (PGE_2, 1 and 10 μM, Cayman, #14010), or Il-33 (10 and 50 ng·mL^{-1}) for further analysis.

siRNA transfection

The small interfering RNA (siRNA) for mouse Egr1 and nonspecific siRNA were designed and synthesized by Ribobio Co., Ltd. (Guangzhou, China). The Egr1 siRNA was transfected into the stromal cells according to the lipofectamine 2000 instructions (Invitrogen). After transfected for 6 h, the stromal cells were treated with Osm under *in vitro* decidualization for 6 h. Transfected cells were analyzed for Real-time PCR.

Real-time PCR

Total RNAs were extracted from the whole uteri or cultured cells using TRIZOL (TaKaRa). cDNA was reverse-transcribed with the RNAs by PrimeScript reverse transcriptase reagent kit (Vazyme, Nanjing, Jiangsu, China) according to the manufacturer's recommendations. Real-time PCR was performed using a SYBR Premix Ex Taq kit (Vazyme) on the CFX96 Touch™ Real-Time System (Bio-Rad). Data were analyzed using the $2^{\triangle\triangle Ct}$ method and normalized to *rpl7* level. The corresponding primer sequences of each gene are provided in Table 1.

Flow cytometry

Flow cytometry was performed as previously described [3]. In brief, the uterine endometrial stromal cells were fixed in 70% ice-cold alcohol at 4 °C overnight. After cells were

centrifuged and washed in ice-cold PBS three times, cells were treated with 1 μL RNase (TaKaRa) in 100 μL PBS at 37 °C for 30 min. Thereafter, cells were stained with 6 μL propidium iodide (Sigma) in 200 μL PBS on ice. Data from flow cytometry were used to analyze the G0/G1, S and G2/M phases of cell cycles by the MODFIT software (Verity Software House, ME, USA).

Western blot analysis

Western blot was performed as described previously [19]. Briefly, proteins were extracted from cultured cells or endometrial tissues by homogenization lysis buffer. The protein lysate samples separated by 10% SDS/PAGE and transferred onto a PVDF membrane followed with blocking in 5% nonflat milk for 1 h. PVDF membranes were incubated at 4 °C overnight with the corresponding primary antibodies for p-Stat3 (Cell Signaling Technology, #9145, Danvers, MA, USA), Egr1 (Cell Signaling Technology, #4153), Lamin A/C (Cell Signaling Technology, #2032), tubulin (Cell Signaling Technology, #2144) or integrin β3 (Cell Signaling Technology, #13166). Thereafter, membranes were incubated with the HRP-conjugated secondary antibody (1 : 5000) in 5% nonfat milk for 1 h. Signals were detected by ECL Chemiluminescent kit (Millipore, San Francisco, CA, USA).

Preparation of nuclear and cytoplasmic extracts

The extraction of nuclear and cytoplasmic extracts was carried out as described previously [19].

Statistical analysis

Data were presented as the mean ± standard deviation unless stated otherwise. Two groups were compared by Student's *t* test. One-way ANOVA followed by Student's *t*-test was performed for multiple comparisons. At least three independent repeats were performed for all of the experiments. $P < 0.05$ was considered to be significantly different between groups.

Results

In situ hybridization localization of *Osm* and *Osmr* mRNA expression in mouse uterus during early pregnancy

In situ hybridization was used to localize the expression of *Osm* and *Osmr* mRNA in mouse uteri from days 1 to 8 of pregnancy. From days 1 to 4, *Osm* signal was mainly located in the uterine glandular epithelium and luminal epithelium. With the onset of implantation on day 5, *Osm* was highly expressed in the stromal cells surrounding the blastocyst. From days 6 to 8, *Osm* was expressed in the decidual stromal cells (Fig. 1A). *Osmr* was similar to *Osm*, but the signals were relatively weaker. From days 1 to 4, there was a weak signal of *Osmr* in the uterine glandular epithelium and luminal epithelium. From days 5 to 8, *Osmr* signals were detected in the decidual zone surrounding the blastocyst. In the control groups, there were no detectable signals for Osm or Osmr from days 1 to 8 (Fig. 1A).

Real-time PCR was used to quantify the expression levels of *Osm* and *Osmr* mRNA expression in mouse uterus on days 5 and 8 of pregnancy. The levels for both *Osm* and *Osmr* mRNA at the ISs were significantly higher than that at inter-ISs on days 5 and 8, respectively (Fig. 1B–E).

Immunohistochemistry data showed that the signal for Osm protein was mainly located in the luminal epithelium and glandular epithelium from days 1 to 4, accompanied by a gradual weakening of the signal from days 1 to 4. On day 5, the signal was seen in the luminal epithelia and the stromal cells surrounding the blastocyst. From days 6 to 8, Osm signal was detected in the decidual area around the blastocyst (Fig. 1F). In general, the pattern of *Osm* mRNA expression was similar to that of Osm protein from days 1 to 8.

Effect of Osm on embryos implantation and two-cell embryos development

To explore the role of Osm in embryo implantation, Osm-soaked blue beads similar to embryo size were used to mimic the action of mouse blastocysts. Compared to control, there were more blue sites similar to embryo attachment when Osm-soaked beads were transferred into the uterine lumen of day 4 pseudopregnant mice (Fig. 2A,B).

In order to answer whether Osm mainly acted on the uterus or embryos, two-cell embryos were cultured to blastocyst stage with Osm-containing medium. There was no detectable difference for the rate of embryo development to blastocyst stage between control and Osm-containing medium (Fig. 2C,D), suggesting the beneficial effect of Osm on embryo implantation was mainly from the uterus.

Effect of Osm on the implantation reaction

Because Osm could initiate attachment reaction, we would like to analyze how Osm regulated embryo implantation. However, p-Stat3 and integrin β3 are key mediators for mouse implantation [20,21]. Western blot data showed that both p-Stat3 and integrin β3

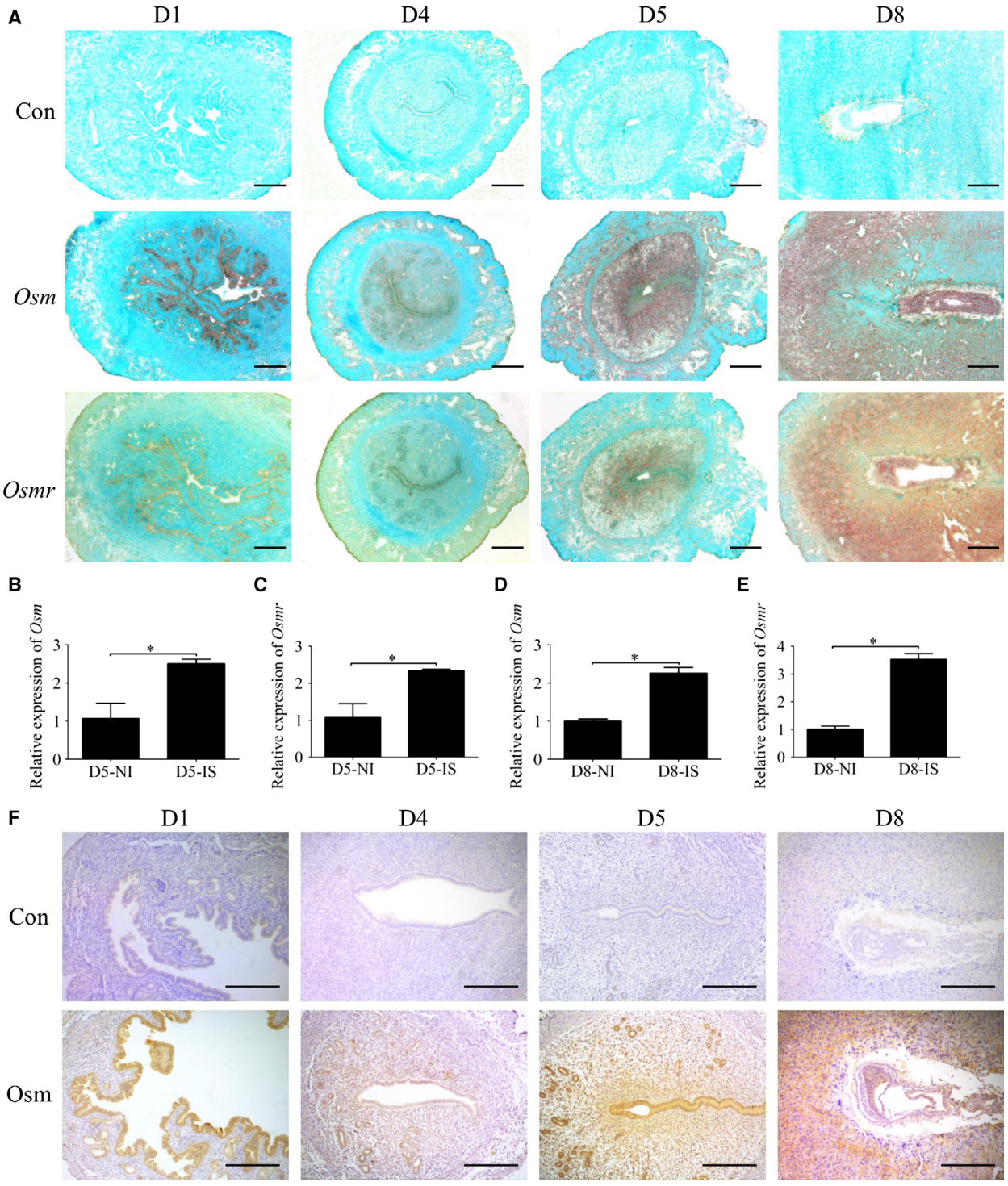


Fig. 1. *Osm* and *Osmr* expression in mouse uteri during early pregnancy. (A) *In situ* hybridization localization of *Osm* and *Osmr* mRNA in mouse uteri on days 1 (D1), 4 (D4), 5 (D5), and 8 (D8). A sense probe was provided as a negative control. Real-time PCR results show the mRNA levels of *Osm* (B) and *Osmr* (C) at implantation site (IS) and inter-implantation site (NI) on day 5, and the mRNA levels of *Osm* (D) and *Osmr* (E) on day 8. (F) Immunohistochemistry of Osm protein in mouse uteri on days 1 (D1), 4 (D4), 5 (D5), and 8 (D8). Bar, 300 μm. *, $P < 0.05$.

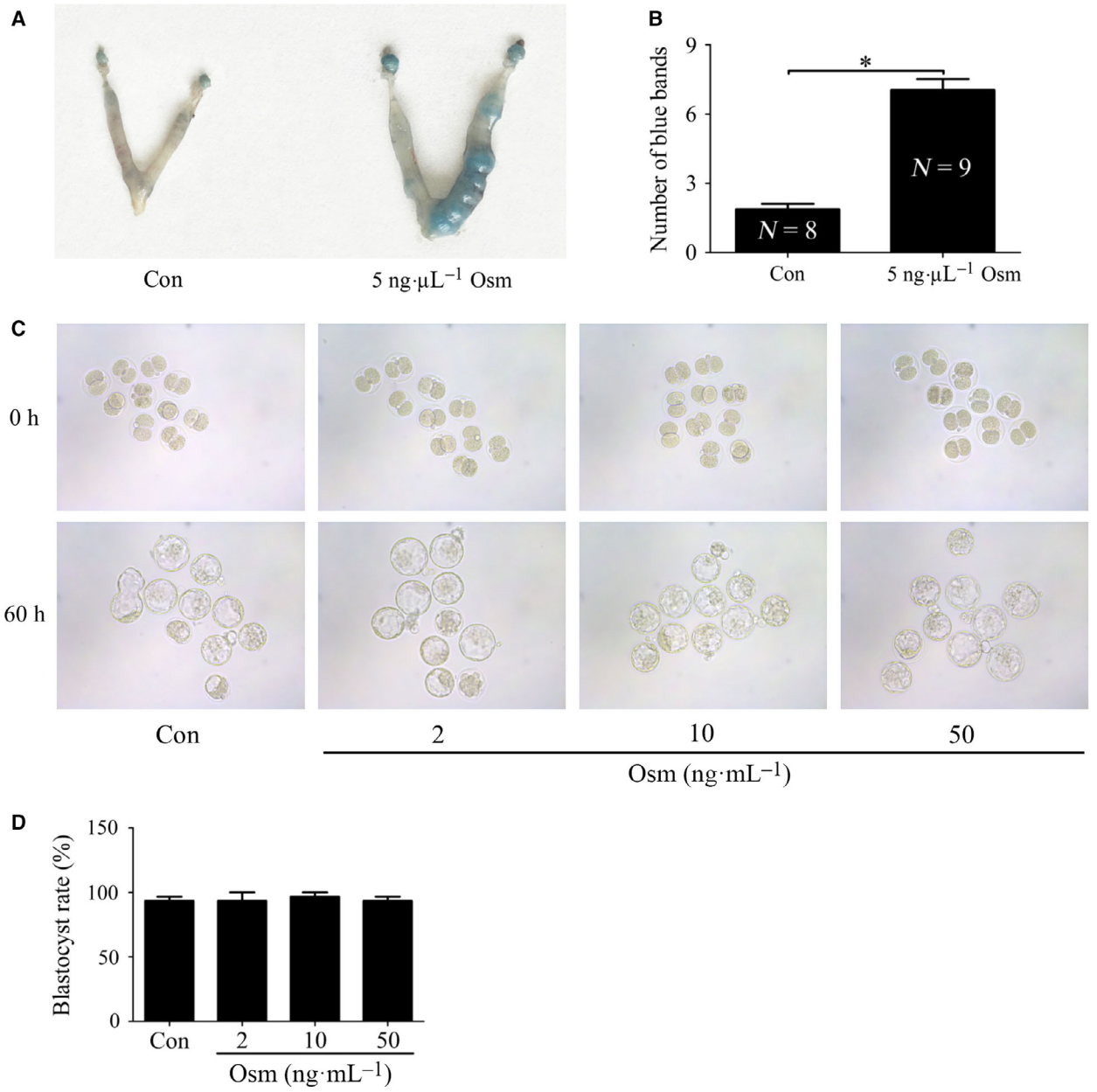


Fig. 2. Effects of Osm on embryos implantation and the development of two-cell embryos. (A) The blue sites on day 6 after 0.1% BSA (left) and 5 ng·μL^{-1} OSM-soaked blue beads (right) were transplanted into uterine lumen on day 4 pseudopregnancy. (B) The number of blue sites following the transfer of 0.1% BAS or Osm-soaked beads. (C) Effects of Osm on the development of two-cell embryos developed to blastocysts. Embryos at the two-cell stage were cultured with 2, 10, and 50 ng·mL^{-1} Osm, respectively. BSA was used as control. Three independent experiments were performed. (D) The ratio of blastocysts. *, $P < 0.05$.

levels were up-regulated after cultured luminal epithelial cells were treated with Osm for 24 h (Fig. 3A). Il-33, a member of Il-1 family, is shown to be essential for mouse embryo implantation and induced in lung epithelial cells by Osm [22,23]. When cultured uterine epithelial cells were treated with Osm, both *Il-33* and *Osmr* mRNA expressions were extremely stimulated (Fig. 3B,C). Meanwhile, the level of integrin β3 protein increased after uterine epithelial cells were treated with Il-33 (Fig. 3D).

Because integrin β3 was induced by Il-33, embryo attachment assay was used to examine whether Il-33 was involved in embryo attachment. After uterine epithelial cells were treated with Il-33, the rate of

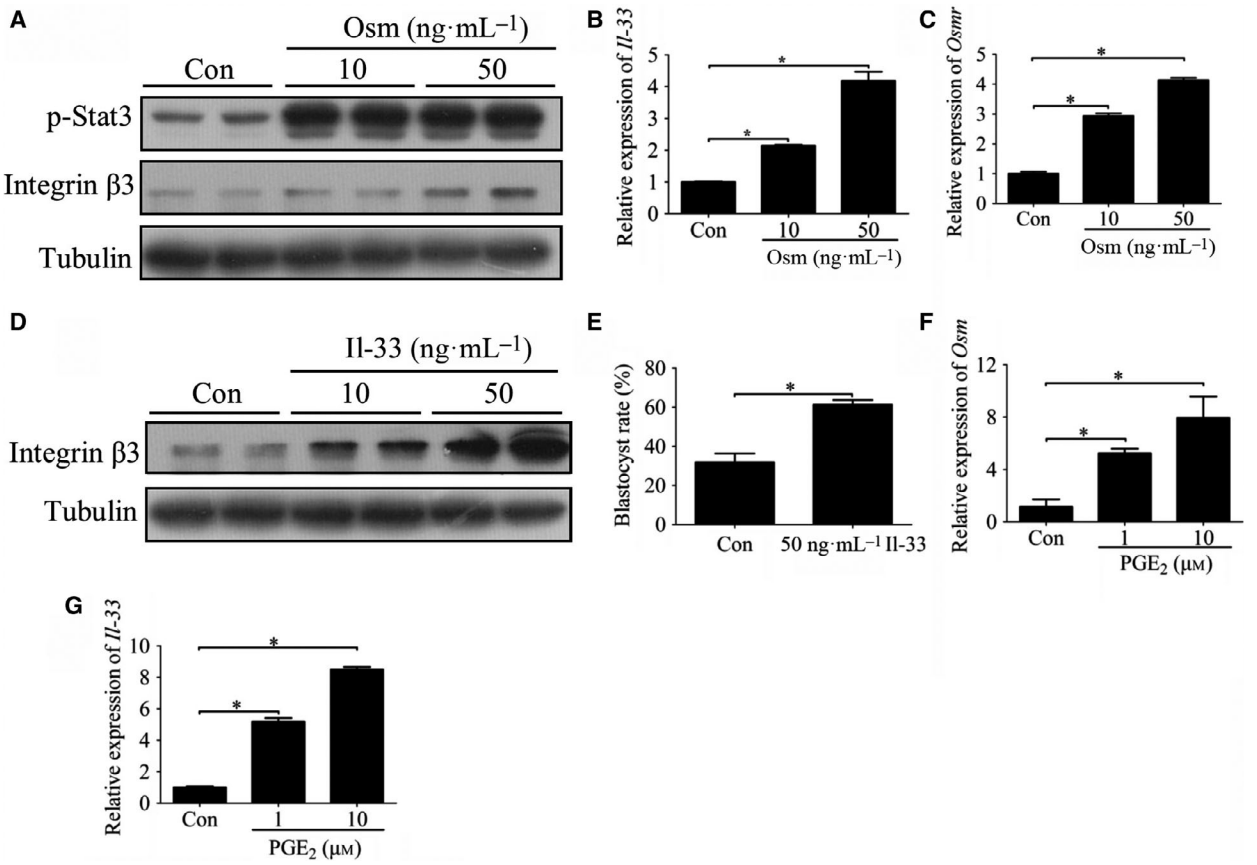


Fig. 3. Effects of Osm during embryo implantation. (A) Western blot of p-Stat3 and integrin β3 after mouse endometrial epithelial cells were treated with Osm for 24 h. (B) The mRNA expression of *Il-33* after cultured epithelial cells were treated with Osm for 3 h. (C) The mRNA expression of *Osmr* after cultured epithelial cells were treated with Osm for 3 h. (D) Western blot of integrin β3 protein level after cultured epithelial cells were treated with Il-33 for 24 h. (E) The ratio of blastocyst attachment on epithelial cells after epithelial cells were treated with Il-33. (F) The level of *Osm* mRNA expression after cultured epithelial cells were treated with PGE_2 for 3 h. (G) The *Il-33* mRNA level after the epithelial cells were treated with PGE_2 for 3 h. *, $P < 0.05$.

blastocyst attachment to cultured epithelial cells was significantly higher compared to control (Fig. 3E).

Our present data showed that Osm from luminal epithelium may regulate embryo implantation *via* Il-33. We wondered how embryos regulate Osm expression during embryo implantation. Among the factors secreted from blastocysts, PGE_2 is essential for embryo implantation and decidualization [24–26]. After cultured epithelial cells were treated with PGE_2, the levels of both *Osm* and *Il-33* mRNA were significantly increased (Fig. 3F,G).

Effects of Osm on *in vitro* decidualization

Because *Osm* was also strongly expressed in decidual cells from days 5 to 8 of pregnancy, we would like to explore whether Osm was involved in decidualization. When stromal cells under *in vitro* decidualization were treated with Osm for 24, 48 or 72 h, Osm could dose-dependently stimulate the mRNA levels of *Dtprp*, *Abpl* and *E2f8* (Fig. 4A–C, data for 24 and 48 h no shown), which were the markers for *in vitro* decidualization [3,27,28]. Furthermore, *Dtprp* mRNA level was also increased in a dose-dependent manner when cultured stromal cells were treated with Osm for 24, 48 or 72 h without *in vitro* decidualization (Fig. 4D, data for 24 and 48 h no shown).

Because polyploidy is a feature of mouse decidualization [29], flow cytometry was performed to analyze the cell cycle after stromal cells under *in vitro* decidualization were treated with Osm. Osm-treated stromal cells showed a significant increase in G2/M phase compared to control (Fig. 4E), suggesting that Osm could induce the polyploidization of stromal cells *in vitro*

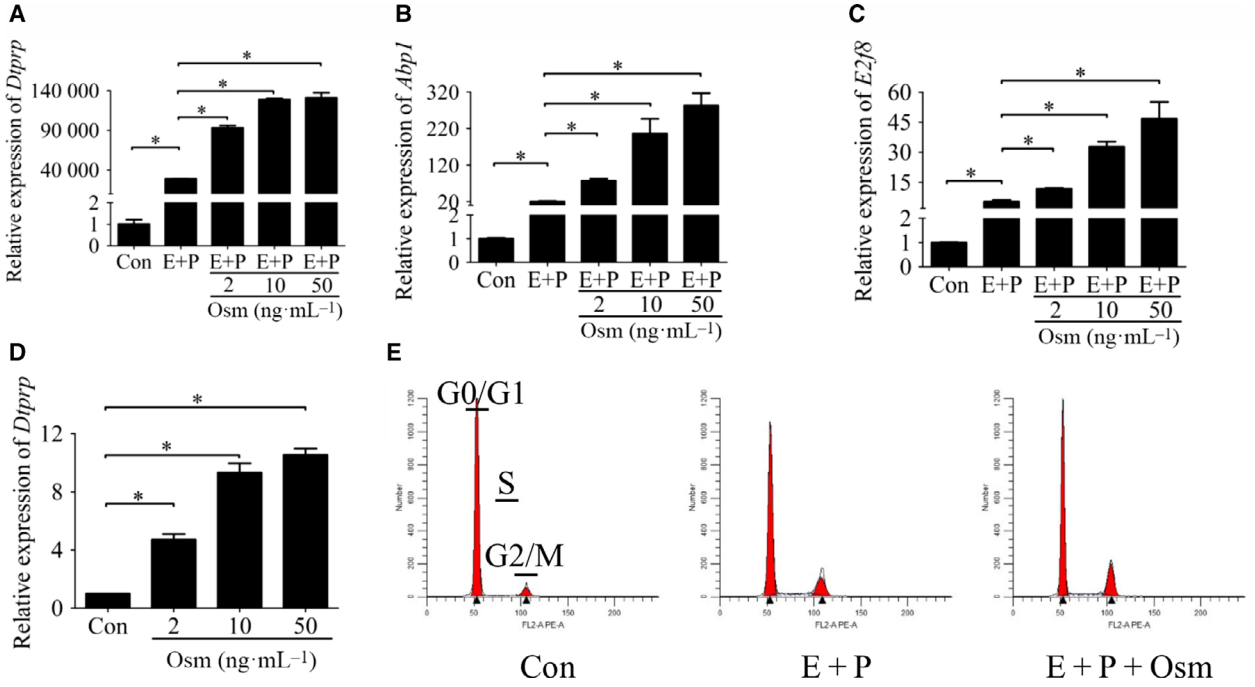


Fig. 4. Effects of Osm on *in vitro* decidualization. The mRNA levels of *Dtprp* (A), *Abp1* (B) and *E2f8* (C) after the stromal cells were treated with Osm for 72 h under *in vitro* decidualization. (D) The *Dtprp* level after the stromal cells were treated with Osm for 72 h. (E) Flow cytometry analysis of cell cycle distribution after stromal cells were treated with Osm for 48 h under *in vitro* decidualization. Con, control group. E + P, E2 and P4. *, $P < 0.05$.

Effects of Osm on the expression of Egr1

Our previous study showed that *Egr1* is strongly expressed in stromal cells at the early IS and important for decidualization [30]. We found *Osm* expression pattern was similar to *Egr1* in the stromal cells at ISs. We wondered whether there was an interaction between Osm and Egr1. Under *in vitro* decidualization, both *Dtprp* and *Egr1* mRNA levels were up-regulated when stromal cells were treated with Osm for 6 h (Fig. 5A,B). *Osmr* mRNA level was also stimulated by Osm treatment, similar to epithelial cells (Fig. 5C). Under *in vitro* decidualization, Osm-induced increase of *Dtprp* mRNA was significantly reduced by Egr1 siRNA (Fig. 5D,E). These results showed that the induction of decidualization by Osm may be regulated through Egr1.

Because Stat3 is a key downstream target of Osm signaling, we assumed that phosphorylated Stat3 may mediate the regulation of Osm on Egr1. Under *in vitro* decidualization, Osm-stimulated increase in *Dtprp* level was down-regulated when these cells were treated with the inhibitor of Stat3 (Fig. 5F). After stromal cells were treated with Osm, there was a significant increase of p-Stat3 in both nuclear and cytoplasmic extracts, p-Stat3 signal in nuclear extract being obviously stronger than the cytoplasmic signal (Fig. 5G). Meanwhile, Osm-induced increase in Egr1 protein was reduced by S3I (Fig. 5H). Our results showed that Osm may regulate *in vitro* decidualization *via* Stat3-Egr1 pathway.

Discussion

Our results showed that Osm is mainly expressed in the glandular and luminal epithelia pre-implantation and strongly expressed in the stromal cells around the embryo and gradually to the decidualization area after implantation, which is similar to Osm localization in a previous study on days 5 and 6 of pregnancy [12,13]. Osm acts mainly through Osmr and gp130 [31]. Osmr is also expressed in mouse uterus at IS, similar to Osm pattern. Gp130 is strongly expressed in the stromal cells after embryo implantation [32,33]. Our data suggest that there is a colocalization for Osm, Osmr and gp130 in mouse uterus at IS.

In our study, blue reaction of embryo attachment can be initiated by Osm-soaked blue beads. p-Stat3 and integrin β3, key implantation markers, are also upregulated by Osm. These data suggest that Osm should be essential for embryo implantation. Maternal Lif is required for embryo implantation [9]. In Lif-null

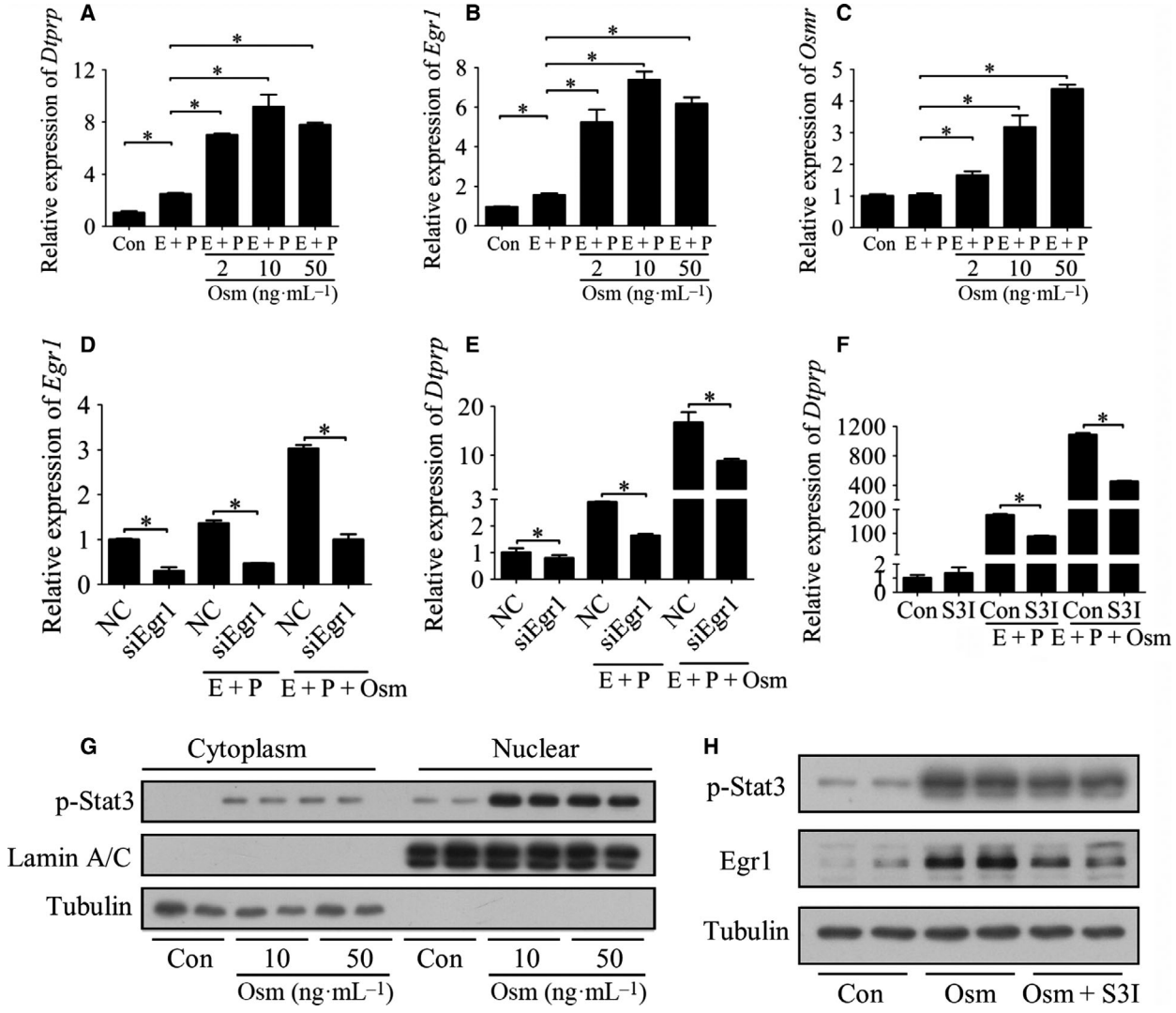


Fig. 5. Effects of Osm on the expression of Egr1 and p-Stat3 during *in vitro* decidualization. (A) The mRNA levels of *Dtprp* (A), *Egr1* (B) and *Osmr* (C) after the stromal cells were treated with Osm for 6 h under *in vitro* decidualization. The mRNA levels of *Egr1* (D) and *Dtprp* (E) after stromal cells were treated with Egr1 siRNA or 10 ng·μL^{-1} Osm for 6 h. (F) Effects of 100 μM S3I on Osm-induced *Dtprp* mRNA expression. (G) The levels of p-Stat3 in both cytoplasmic and nuclear extracts after the stromal cells were treated with Osm for 24 h. (H) Effects of 100 μM S3I on Osm-induced increase in Egr1 and p-Stat3. E + P, *in vitro* decidualization. Tubulin and Lamin A/C were used as the internal reference of cytoplasm and nuclear, respectively. *, $P < 0.05$.

mice, Osm expression is absent compared to control [12]. Gp130, a key molecular in Osm receptor complex is also required for embryo implantation because uterine gp130 deletion leads to implantation failure [34]. Based on these data, Osm should play a role during embryo implantation *via* gp130 and Osmr.

Our study indicated that Osm may play a role during embryo implantation. We are wondering how Osm acts on embryo implantation. PGE_2 can be synthesized and secreted by mouse blastocysts [35]. In our study, Osm expression is stimulated by PGE_2. Both EP2 and EP4 of PGE_2 receptors are localized in the luminal epithelium in mice [36,37]. It is possible that blastocyst-released PGE_2 may induce Osm expression in luminal epithelium. Il-33 has been shown to be important for mouse implantation [23]. In macrophages, PGE_2 is able to stimulate Il-33 production [38]. We showed PGE_2 can induce the Osm and Il-33 expression *in vitro* mouse luminal epithelial cells. Meanwhile, Osm also can improve the expression of Il-33 and phosphorylated Stat3. Therefore, PGE_2 from blastocysts may promote embryo implantation *via* Osm and Il-33.

During *in vitro* decidualization, our data showed that Osm can stimulate the expression of *Dtprp, Abp1, E2f8*, the marker molecules of decidualization *in vitro* [3,28,39]. E2f8 is a marker for polyploidization during mouse decidualization [3]. Our flow cytometry results also showed that Osm treatment increases the rate of polyploidization in stromal cells. *Egr1* is strongly expressed in stromal cells in the ISs [30], and shares a similar localization with *Osm*. In our study, Osm can induce *Egr1* expression under *in vitro* decidualization. In addition, Egr1 knockdown abrogates the Osm-induced up-regulation of *Dtprp*. Because the inhibitor of Stat3 is able to block Osm-stimulated increase of Egr1, Osm may regulate mouse decidualization through Stat3-Egr1 pathway.

In conclusion, our study shows that PGE_2-induced Osm may mediate embryo implantation through Il-33 and participate in decidualization *via* Egr1.

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Author contributions

TF and ZMY designed study, analyzed data and wrote paper; TF, HTZ, and HYZ performed animal work, cell culture and qPCR; ZCC and BL performed embryo culture; all authors gave the final approval of this paper.

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