Role of osteopontin in decidualization and pregnancy success

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Abstract

OPN is essential for blastocyst implantation and placentation. Previous study found that miR181a was increased while miR181b was downregulated in endometrium during decidualization. However, the information regarding their effects on decidualization in human endometrium is still limited. Here, we report a novel role of OPN and miR181b in uterine decidualization and pregnancy success in humans. The expression of OPN was high in endometrium in secretory phase and *in vitro* decidualized hESC, whereas miR181b expression was low in identical conditions. Further analysis confirmed that OPN expression was upregulated by cAMP and C/EBP β signal pathway, while downregulated by miR181b. Increased OPN expression could promote the expression of decidualization-related and angiogenesis-related genes. Conversely, the processes of decidualization and angiogenesis in hESC were compromised by inhibiting OPN expression *in vitro*. OPN expression was repressed in implantation failure group when compared with successful pregnancy group in IVF/ICSI-ET cycles. These findings add a new line of evidence supporting the fact that OPN is involved in decidualization and pregnancy success.

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Introduction

Embryo implantation is a highly regulated process that involves in a dialogue between the competent blastocyst and the receptive endometrium. When the blastocyst implants into the uterus, the endometrial stromal cells undergo extensive proliferation, differentiation, vascular remodeling and immune cell recruitment, a process known as decidualization, which is essential for successful pregnancy (Gellersen *et al.* 2007). The decidual tissue forms an immune tolerance environment for the allograft embryo and provides crucial secretory factors for nourishing the embryo before the maturation of placenta. It has been well established that defects in decidualization will lead to reproductive disorders, such as infertility, spontaneous abortion, preeclampsia, fetal growth retardation (Cha *et al.* 2012).

Successful embryo implantation requires the intricate regulatory network of cytokines, chemokines, growth factors and immune cells. Osteopontin (OPN), a member of extracellular matrix (ECM) protein family, is involved in many physiological and pathological processes, including cell adhesion, cell proliferation and differentiation, angiogenesis and tumor metastasis. Microarray screening showed OPN expression is upregulated 8- to 12-fold in total endometrium between the early and mid secretory phase, suggesting it may serve as a marker of receptivity (Carson *et al.* 2002,

Kao *et al.* 2002, Ruiz-Alonso *et al.* 2012, Bhagwat *et al.* 2013, Diaz-Gimeno *et al.* 2014). Immunohistochemistry displayed a strong staining of OPN in glandular epithelial cells and decidualized stromal cells (von Wolff *et al.* 2004, Franchi *et al.* 2008). Besides, previous studies also revealed OPN expression in stromal cells is increased at the end of the secretory phase, which is consistent with the time when perivascular stromal cells begin to decidualize (Apparao *et al.* 2001, von Wolff *et al.* 2004). OPN-deficient mice manifested a decreased pregnancy rate during mid-gestation and knockdown of OPN in mouse endometrial stromal cells lead to a restrained trophoblast invasion *in vitro* (Weintraub *et al.* 2004, Qi *et al.* 2014). Our previous study also proved that OPN is activated in mouse endometrial stromal cells (mESC) and human endometrial stromal cells (hESC) by estrogen (E2) and progesterone (P4) (von Wolff *et al.* 2004, Qi *et al.* 2014). These results suggested that OPN plays an important role in regulating blastocyst implantation and decidualization. However, the regulation and function of OPN on ESC proliferation, differentiation and vascular remodeling remains unknown.

Cyclic adenosine monophosphate (cAMP) is a second messenger molecule that is vital for decidualization (Gellersen & Brosens 2003). CCAAT enhancer-binding protein β (C/EBP β), a transcription factor belongs to a C/EBP family, regulates the expression of insulin-like growth factor-binding protein (IGFBP-1) and

prolactin (PRL) during decidualization (Tamura *et al.* 2014). Promoter analysis found that there exists a C/EBP β binding site on the promoter of OPN. MicroRNAs (miRNAs) are small noncoding RNAs that are important determinants in the post-transcriptional regulation. miRNA microarray analysis and miRNA PCR array revealed that miR181b was downregulated in decidualized ESCs when compared to non-induced ESCs (Qian *et al.* 2009, Estella *et al.* 2012), indicating that miR181b is involved in decidualization. OPN may be a target gene of miR181b according to the results from TargetScan online program (Chen *et al.* 2016). Therefore, we hypothesized that the mutual regulation between OPN and miR181b may contribute to embryo implantation and decidualization.

In this study, we demonstrated that OPN is upregulated in human endometrium during secretory phase and decidualized hESCs. We investigated the regulation of cAMP and C/EBP β on OPN and the effects of OPN on decidualization and angiogenesis in hESCs. Our results revealed that miR181b expression is decreased during decidualization and overexpression of miR181b will inhibit the expression of OPN in hESC. Besides, OPN is significantly repressed in the failed group when compared with successful pregnancy group in *in vitro* fertilization/intracytoplasmic sperm injection-embryo transfer (IVF/ICSI-ET) cycles. These findings add a new line of evidence supporting the fact that OPN is required for decidualization and pregnancy success.

Materials and methods

Experimental design

In cell culture and treatment experiments, there were three duplicates in each group, and the treatments were repeated three times. For the detection of OPN in different menstrual cycle phase, seven endometrium samples in proliferative phase (from day 5 to day 14 of menstrual cycle), six in early secretory phase (from day 15 to 19 of menstrual cycle), seven in mid-secretory phase (from day 20 to 24 of menstrual cycle) and five in late-secretory phase (from day 25 to 29 of menstrual cycle) were included in this study. For evaluating the relationship between OPN and fertility, 10 patients with successful clinical pregnancy and 10 patients with implantation failure were included in this study. The patients with endocrine diseases, hormonal drugs history, intrauterine adhesion, repeated implantation failure and repeated spontaneous abortion and gynecological tumor were excluded in this study.

Cell culture and *in vitro* decidualization

The immortalized human endometrial stromal cell line (hESC, CRL-4003) was cultured in DMEM/F12 growth media with 2% charcoal-stripped fetal calf serum, 1% antibiotics and puromycin in an atmosphere of 5% CO₂ at 37°C. The hESC cells were decidualized *in vitro* by incubating the cells with

10 nM estradiol-17 β (E, Sigma), 1 μ M progesterone (P, Sigma) and 0.5 mM cAMP (Sigma) for 8 days. The medium was replaced every 48 h. PRL and IGFBP-1 were used as makers for decidualization.

Endometrial samples

The study was approved by the Ethical Research Committee of Renmin Hospital of Wuhan University, and all patients included in the study signed a written informed consent. The endometrial samples were collected from patients who received curettage or hysteroscopy at Renmin Hospital of Wuhan University. Proliferative phase ($n=7$), early secretory phase ($n=6$), mid-secretory phase ($n=7$) and late secretory-phase ($n=5$) samples were obtained in cycles in which the cycle was identified by endometrium biopsy examination. The IVF/ICSI patients took the serum hormone detection and transvaginal ultrasound examination on day 21 or day 22 of menstrual cycle to determine the ovulation occurrence (elevated P level and corpus luteum formation). If the ovulation is confirmed, the patients will receive GnRH-agonist downregulation treatment, ovulation induction, IVF/ICSI and embryo transfer. The endometrium samples were collected on day 21–22 of menstrual cycle by curettage if the patient met the above mentioned criteria. The patients will take serum hCG examination 12 days after embryo transfer and ultrasound examination 30 days after transfer. The patients were divided into successful pregnancy group ($n=10$) and failed implantation group ($n=10$) according to hCG and ultrasound results. Samples were stored at -70°C or fixed in 10% formalin and embedded in paraffin.

RNA extraction and real-time PCR

Total RNAs from cultured cells and tissues were extracted using TRIzol (TaKaRa Bio) following the manufacturer's recommendations. Small RNA was extracted using RNAiso for Small RNA (TaKaRa Bio). RNA was reverse transcribed into cDNA using the PrimeScript RT reagent Kit with gDNA Eraser or Mir-X miRNA First-Strand Synthesis Kit (TaKaRa Bio). cDNA for real-time PCR was amplified using a SYBR Premix Ex Taq II kit (TaKaRa Bio), performing on the ABI 7500 Fast Real-time PCR System (Applied Biosystems). GAPDH served as an internal control and all reactions were run in triplicate. The relative expression levels of all target genes were calculated using the $2^{-\Delta\Delta\text{CT}}$. The sequences of real-time PCR primers are listed in Table 1.

Western blot analysis

Tissues and cultured cells proteins were extracted using lysis buffer supplemented and protein concentrations were measured using BCA Protein Assay Kit. Protein was loaded at the concentration of 1000 ng/well on SDS-polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Millipore). Membranes were blocked with 5% skim milk for 1 h at room temperature. Membranes were incubated with primary antibodies in 5% BSA (1:1000) as follows: mouse monoclonal

Table 1 Primer sequences for real-time PCR.

Target mRNA	Forward	Reverse
GAPDH	GAAGGTGAAGGTCGGAGT	GATGGCAACAATATCCACTT
PRL	AAGCTGTAGAGATTGAGGAGCA	TCAGGATGAACCTGGCTGACTA
IGFBP1	CCAAACTGCAACAAGAATG	GTAGACGCACCAGCAGAG
OPN	ATGGAAAGCGAGGAGTTG	ATCTGGACTGCTTGTGGC
VEGFA	CACTCAGATCCTGACAGGGAA	GCTGGGTTTGTCCGGTGT
VEGFR2	CAAGACAGGAAGACCAAGAAAAGAC	GGTGCCACACGCTCTAGGA
miR181a-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACTCACC	
miR181b-5p	TGAACATTTCATTGCTGTCCGGTG	
U6	CGCTTCGGCAGCACATATAC	TTCACGAATTTGCGTGTCTCAT

OPN antibody (Santa Cruz Biotechnology, sc-21742), mouse monoclonal insulin-like growth factor-binding protein-1 (IGFBP-1) antibody (Santa Cruz, sc-25257), mouse monoclonal vascular endothelial growth factor A (VEGFA) antibody (Santa Cruz, sc-365578) and rabbit monoclonal beta-ACTIN antibody (Cell Signaling Technology, 4970) overnight and washed. Then membranes were incubated with horseradish peroxidase-labeled anti-mouse or anti-rabbit IgG secondary antibody in 5% skim milk (1:4000) at room temperature for 1 h and visualized by enhanced chemiluminescence.

Immunohistochemistry

All the paraffin-embedded tissues were de-paraffinized and re-hydrated. The tissues were then incubated in 3% H₂O₂ to inactivate endogenous peroxidase. Nonspecific binding sites were blocked with normal goat serum at room temperature. After serum was removed, tissue was incubated with primary mouse monoclonal OPN antibody (1:400 dilution, Santa Cruz, sc-21742) in 10% goat serum or rabbit Ig G (1:400 dilution, Santa Cruz) at 4°C overnight, respectively. After washing in PBS, the sections were incubated with HRP-conjugated secondary antibodies (1:200 dilution, ZSGB-BIO, Beijing, China) in 1% BSA. The color was developed with a DAB kit (Vector Laboratories). Positive signals of OPN were visualized as brown in color. Afterwards, the samples were studied under a microscope (DM2500, Leica) and digitized to 100× or 400× magnification (LAS software V4.3, Leica). Five randomized fields were observed under the microscope for each IHC slice.

Plasmid construction

Short hairpin RNA (shRNA) sequences were designed and synthesized by TSINGKE Biological Technology (Wuhan, China). The shRNA sequences were annealed and ligated with linearized Plko.1 puro vector. The sequences targeting OPN and OPN-shNC are GAGCAATGAGCATTCCGATGT and GTTCTCCGAACGTGTACGT respectively. The sponge sequence, encoding ten repeats of reverse complementary sequence of mature has-miR-181b was synthesized by Sunny Biotechnology Co. (Shanghai, China). The miR-181b-sponge sequence was ligated to the pHX511 vector after digestion by NotI and XhoI. To construct OPN, C/EBPβ and miR181 overexpression vectors, OPN, C/EBPβ and miR181 were amplified from hESCs cDNA or genomic DNA by PCR respectively. Then, these sequences were

ligated into pcDNA5-Flag, pcDNA3.1 or PLemiR vector. The sequences for overexpression were as follows: OPN sense: 5'-CGGGATCCATGAGAATTGCAGTGATTTC-3'; OPN antisense: 5'-CCGCTCGAGTTAATTGACCTCAGAAGATG-3'; C/EBPβ sense: 5'-AGGCGAATTCG-CAACGCCTGGTGGC CTGGGACCCA-3'; C/EBPβ antisense: 5'-CGGTCTCGAG CTAGCA-GTGGCCGGAGGAGGCGAGC-3'; miR181b sense: 5'-CCG CTGAGCCTGTGCAG-AGATTATTTT-3'; miR181b antisense: 5'-ATAAGAATGCGGCCGCAAGCG-GGGCC ACAGTTGCAT. All plasmids were sequenced to verify the inserted targets.

The OPN promoter fragment was amplified with prime as follows: sense: 5'-GACGCGTCCGCTTATGCGGGTATGTTT-3'; antisense: 5'-CCAAGCTTGGTTCCTTG-GTCCGGCGTTTGG-3'. For the generation of the luciferase reporter construct, the OPN promoter fragment was ligated into luciferase reporter vector pGL3-Basic.

Transient transfection

Transfections of hESC were performed with Lipofectamine 2000 (Invitrogen) according to the instructions. Specifically, short hairpin RNA (shRNA) that target to OPN, pcDNA3.1 (+) plasmid, OPN overexpression or C/EBPβ overexpression vectors were transferred into hESCs. The efficiency of transfection was tested by Western blot and real-time PCR after 48-h transfection.

Luciferase assay

The promoter sequence of human OPN was amplified by PCR from human genomic DNA. The amplified products were digested by restriction enzymes and inserted into the pGL3-basic vector upstream from the start codon of luciferase. To determine the effect of C/EBPβ on OPN promoter, hESC were transfected with pGL3-OPN and pRL-TK in combination with pcDNA3.1 (+) or C/EBPβ overexpression vector, respectively. Cells were harvested and examined for luciferase activity 2 days after transfection. Luciferase activity was measured using the dual luciferase assay system (Promega). OPN luciferase activity was normalized with Renilla luciferase activity.

Statistical analysis

Statistical analyses were performed using SPSS statistical software (version 19.0). All the experiments were

independently repeated at least three times. All results are presented as means $\pm$ s.d. ANOVA was used for comparison of multiple variables, and the Student's *t*-test was used to analyze differences in the gene expression between groups. A value of $P < 0.05$ was considered statistically significant, and $P < 0.01$ represented sufficient statistical significance.

Results

Expression of OPN and miR181a/b in human endometrium

We detected the expression pattern of OPN protein in human endometrium by immunohistochemistry. OPN protein was barely detectable in human endometrium during proliferative phase. The signal intensity of OPN protein was gradually increased during secretory phase, OPN protein was mainly localized at glandular epithelium and decidualized stromal cells, the IgG was used as negative control (Fig. 1A). Real-time PCR and Western blot were performed to examine the expression level of OPN in human endometrium. As shown in Fig. 1B and C, the mRNA and protein level of OPN in secretory endometrium was significantly upregulated when compared with proliferative phase. In order to evaluate the regulation network of miR181a, miR181b and OPN, we detected the expression of miR 181a and miR181b, the results showed that the expression of miR 181a is constant during proliferative and secretory phase (Fig. 1D), while the expression of miR181b is decreased in secretory phase when compared with proliferative phase (Fig. 1E).

Expression of OPN and miR181b hESCs during *in vitro* decidualization

hESCs were treated with E2, P4 and cAMP to induce decidualization *in vitro*, real-time PCR was performed to quantify the mRNA level of PRL and IGFBP-1 to identify the success of decidualization. PRL and IGFBP-1 mRNA levels were upregulated in decidualized hESC (Fig. 2A and B), accompanied with the upregulation of OPN mRNA and protein (Fig. 2C and E). On the contrary, miR181b level was decreased during decidualization (Fig. 2D).

cAMP and C/EBP β regulate the expression of OPN

The decidualization process is induced by progesterone (P4) on an E2 primed endometrium, mediating through cAMP signaling pathways in humans. To evaluate the regulatory effect of cAMP on OPN, we treated hESCs with E2+P4, cAMP or E2+P4+cAMP, respectively. Data from real-time PCR showed that the expression of PRL and IGFBP-1 could be induced by cAMP alone, which is further strengthened by E2+P4+cAMP

Figure 1 The expression of OPN, miR-181a/b in human endometrium. (A) The protein localization of OPN in human endometrium during proliferative, early secretory, mid-secretory and late secretory phase was detected by immunohistochemistry, IgG was used as negative control for mid-secretory phase (scale bar: 100 μ m). The mRNA (B) and protein (C) level of OPN in human endometrium during proliferative ($N=7$), early secretory ($N=6$), mid-secretory ($N=7$) and late secretory ($N=5$) phase. Real-time PCR was performed to quantify the mRNA level of miR-181a (D) and miR-181b (E) in human endometrium during proliferative and secretory phase. $*P < 0.05$.

(Fig. 3A and B). OPN was significantly activated by cAMP or E2+P4+cAMP, instead of E2+P4 (Fig. 3C). The luciferase activity of OPN promoter was significantly increased by cAMP treatment (Fig. 3D). These results revealed the expression of OPN in hESC is dependent on cAMP signaling pathway. C/EBP β is an important transcriptional regulator in decidualization and involved in cAMP signaling pathway. To study the regulation of C/EBP β on OPN expression, we constructed a C/EBP β overexpression plasmid. The expression of OPN

Figure 2 The expression of OPN and miR-181b in HESC during *in vitro* decidualization induced by E2, P4 and cAMP for 0, 2, 4, 6 and 8 days, respectively. Real-time PCR was performed to quantify the mRNA level of PRL (A), IGFBP-1 (B), OPN (C) and miR181b (D) in HESC during *in vitro* decidualization. (E) Western blot of OPN protein in HESC during *in vitro* decidualization. There were three duplicates in each group, and the experiments were repeated for three times. * $P < 0.05$.

was significantly increased by the increase of C/EBP β (Fig. 3E). Besides, the luciferase activity of OPN promoter was significantly increased by C/EBP β overexpression (Fig. 3F).

The effect of OPN on decidualization

OPN shRNA and OPN overexpression vector were transfected in hESC to study the effect of OPN on decidualization. The efficiency of transfection was tested by real-time PCR and Western blot 48h later after transfection, respectively (Fig. 4A and B). The expression of PRL and IGFBP1 were inhibited by OPN shRNA transfection (Fig. 4C and D). To further confirm the function of OPN in hESC decidualization, OPN-pcDNA5 vector was transfected into hESC. The mRNA and protein level of OPN was elevated by OPN overexpression (Fig. 4E and F). At the meantime, the expression of PRL and IGFBP-1 were boosted by OPN overexpression (Fig. 4G and H).

Figure 3 The regulation of cAMP and C/EBP β on OPN in HESC. The mRNA level of PRL (A), IGFBP-1 (B) and OPN (C) in HESC after E/P and cAMP treatment. (D) The relative luciferase activity of OPN promoter in PGL3 plasmid under cAMP treatment. (E) The relative luciferase activity of OPN promoter in PGL3 plasmid after C/EBP β overexpression. (F) The mRNA level of OPN in HESC after C/EBP β overexpression. There were three duplicates in each group, and the experiments were repeated for three times. * $P < 0.05$.

MiR181b regulates decidualization through OPN in hESC

To assess the relationship between miR181b and OPN in hESC during decidualization, the plemiR-181b and sponge-miR181b were used as mediators for gain- and loss-of-function studies, respectively. The results from real-time PCR verified the efficiency of sponge-miR181b transfection (Fig. 5A). OPN expression was increased in sponge-miR181b-transfected hESCs when compared with control (Fig. 5B and C). Similar to OPN overexpression in hESC, the mRNA levels of PRL and IGFBP-1 were strengthened after sponge-miR181b transfection during *in vitro* decidualization (Fig. 5D and E). On the contrary, miR181b overexpression by plemiR 181 could inhibit the expression of OPN mRNA and protein (Fig. 5F, G and H), the downregulation of OPN by plemiR 181 transfection obstructed the expression of PRL and IGFBP-1 during *in vitro* decidualization (Fig. 5I

Figure 4 The effect of OPN knockdown and overexpression on hESC decidualization. The mRNA (A) and protein (B) expression of OPN in hESC after shRNA transfection. Real-time PCR was performed to quantify the mRNA level of PRL (C) and IGFBP-1 (D) after OPN-shRNA transfection. The mRNA (E) and protein (F) expression of OPN in hESC after OPN-pcDNA5 transfection. The mRNA level of PRL (G) and IGFBP-1 (H) after OPN-pcDNA5 transfection. There were three duplicates in each group, and the experiments were repeated for three times. * $P < 0.05$.

and J). These results proved that miR181b may play a negative regulation effect on OPN expression in hESC during decidualization.

The expression of VEGFA and VEGFR2 is regulated by OPN and miR181b during decidualization

VEGFA is well known for its roles in angiogenesis, endometrial receptivity, placentation and embryo development (Binder *et al.* 2014). Elevated OPN expression has also been found to be related to angiogenesis during tumorigenesis (Zaravinos *et al.* 2012). We explored the relationship between OPN and angiogenic components, VEGFA and VEGFR2 in hESC during *in vitro* decidualization. Real-time PCR results proved that the expression of VEGFA

and VEGFR2 was gradually induced in hESC during decidualization (Fig. 6A, B and C). Knockdown of OPN could prominently repress the expression of VEGFA and VEGFR2 during *in vitro* decidualization (Fig. 6D and E), while the expression of VEGFA and VEGFR2 was enhanced by OPN overexpression (Fig. 6F and G). These results suggested that OPN may be involved in the regulation of VEGFA and VEGFR2 expression.

Abnormal OPN expression in endometrium is related to embryo implantation failure

Embryo implantation failure is the main 'rate-limiting' factor in assisted reproduction techniques. Since OPN is required for embryo implantation and plays a pivotal role in decidualization and angiogenesis, we assumed that abnormal expression of OPN may be a reason of implantation failure during IVF/ICSI-ET in humans. To confirm this hypothesis, we collected the endometrium samples obtained before IVF/ICSI treatment and divided them into successful ($n = 10$) and failed ($n = 10$) pregnancy group after IVF/ICSI and embryo transfer. The clinical data showed no significant differences in age, ovarian hormonal levels, AMH level, number of retrieved oocytes, number of transferred embryo and endometrial thickness between two groups (Table 2). We detected the expression of OPN in successful and failed groups, respectively. The Western blot results showed that the protein level of OPN was significantly lower in the failed group than in the successful group (Fig. 7A and B).

Discussion

Investigation the molecular mechanisms that control the embryo implantation and decidualization will be crucial for improving implantation rates after embryo transfer (Zhao *et al.* 2017). In humans, decidualization is independent of the presence of an implanting blastocyst, it involves the transformation of endometrial stromal cells into specialized secretory decidual cells, recruitment of immune cells, extracellular matrix remodeling and angiogenesis. This process is controlled by complex and well-organized regulatory network to guarantee the success of pregnancy. Here, we found OPN level is increased in human uterine endometrium during secretory phase when the endometrium undergoes spontaneous decidualization, as well as in the process of E2+P4+cAMP-induced hESC decidualization *in vitro*, suggesting that OPN may play functions in human uterine decidualization.

cAMP is a necessary stimulus for human ESC decidualization in addition to estrogen and progesterone. PRL and IGFBP-1 levels, the markers for hESC decidualization, were significantly increased in hESCs when treated with cAMP or E2+P4+cAMP compared with hESCs that treated with E2+P4. Similarly,

Figure 5 The regulation of miR181b on OPN expression and decidualization in HESC. Real-time PCR was performed to quantify the mRNA level of miR181b (A) and OPN (B) after Sponge-miR181 transfection. (C) The protein level of OPN in HESC after Sponge-miR181 transfection. The mRNA level of PRL (D) and IGFBP-1 (E) under E/P/C treatment and Sponge-miR181 transfection. The mRNA level of miR181b (F) and OPN (G) after plentiR181 transfection. (H) The protein level of OPN in HESC under E/P/C treatment and Sponge-miR181 transfection. The mRNA level of PRL (I) and IGFBP-1 (J) under E/P/C treatment and Sponge-miR181 transfection. There were three duplicates in each group, and the experiments were repeated for three times. * $P < 0.05$.

OPN was significantly increased by treating with cAMP or E2+P4+cAMP, rather than E2+P4, indicating the expression of OPN in hESC is regulated by cAMP. However, *in vitro* decidualization induced by E2+P4 treatment requires more than 14 days, 8 days of E2+P4 treatment is insufficient for *in vitro* decidualization without the addition of cAMP, this could be the reason for the inability of E2+P4 on OPN expression. C/EBPβ is found to mediate the effects of cAMP on uterine stromal cell decidualization. The expression of OPN was significantly increased in hESCs by C/EBPβ overexpression, the luciferase activity results confirmed that OPN is activated by cAMP through transcription regulation. These results indicate that cAMP could regulate OPN expression through C/EBPβ signal pathway.

miRNA, a newly identified family of small noncoding RNAs, which cause the degradation of target mRNA or translational inhibition through target mRNA-specific base pairing (Zhao & Srivastava 2007). Published data indicated that aberrant expression of some miRNAs is related with various reproductive disorders, such

as repeated implantation failure, endometriosis and endometrial cancer (Teague *et al.* 2010, Revel *et al.* 2011, Konno *et al.* 2014). miR181a is increased dramatically in decidualized hESCs and played an important role in hESC decidualization *in vitro* by inhibiting Krüppel-like factor 12 (Zhang *et al.* 2015). However, the effect of miR181b on decidualization has not been intensively studied. Previous studies reported that miR181b was downregulated in endometrium during decidualization (Qian *et al.* 2009, Estella *et al.* 2012). Moreover, miR181b expression is significantly decreased in human early pregnancy deciduas compared with that in menstrual endometrium (Wang *et al.* 2016). In this study, we revealed that miR181b expression is decreased in decidual hESCs compared to non-induced hESCs. OPN was upregulated in miR181b-sponge-transfected hESCs and was downregulated in miR181 overexpression hESCs. Here, we concluded that the expression of OPN is negatively regulated by miR181b. MiR181b attenuated the effect of hormones on decidualization induction and vessel remodeling, miR181b-sponge transfection

Figure 6 The regulation effect of OPN on VEGF expression in HESC during *in vitro* decidualization. Real-time PCR was performed to quantify the mRNA level of VEGFA (A) and VEGFR2 (B) in HESC during *in vitro* decidualization. (C) The protein level of VEGFA in HESC during *in vitro* decidualization. The mRNA level of VEGFA (D) and VEGFR2 (E) after shOPN transfection during *in vitro* decidualization. The mRNA level of VEGFA (F) and VEGFR2 (G) after OPN-pcDNA5 transfection during *in vitro* decidualization. There were three duplicates in each group, and the experiments were repeated for three times. * $P < 0.05$.

Table 2 Statistical analysis of clinical parameters for IVF/ICSI patients.

	Failed pregnancy group (n=10)	Pregnancy group (n=10)	P value
Age (year)	30.60±4.97	29.27±3.58	0.47
Basic FSH	6.39±1.38	6.86±1.67	0.48
AMH	3.38±1.41	3.23±1.38	0.81
Length of menstruation cycle	30.40±5.46	29.50±1.58	0.59
E2 on the day of biopsy	197.79±96.05	216.81±93.94	0.57
P4 on the day of biopsy	14.97±5.84	15.20±7.47	0.94
Number of retrieved oocytes	15.50±3.69	14.10±4.65	0.47
Number of transferred embryo	1.90±0.57	2.10±0.32	0.34
Thickness of endometrium on the day of transfer	1.18±0.19	1.15±0.19	0.73

will facilitate the effect of hormones on decidualization induction and vessel remodeling. Taken together, these results proved our hypothesis on the effect of miR181b on OPN expression, decidualization and angiogenesis.

The VEGF family has three receptor tyrosine kinases, VEGFR1, VEGFR2 and VEGFR3. Among the VEGF family, VEGFA is fundamental to endometrial angiogenesis through activating VEGFR2 (Maruyama & Yoshimura 2008, Claesson-Welsh & Welsh 2013). Vessel remodeling is a vital process included in decidualization. OPN and VEGF are characterized by a convergence in function for regulating angiogenesis and knockdown of OPN expression suppresses expression of VEGF (Wu *et al.* 2014, Ramchandani & Weber 2015, Xu *et al.* 2015). In this study, we demonstrated that OPN knockdown could cause the downregulation of VEGFA and VEGFR2, suggesting that OPN may involved in VEGFA and VEGFR2 signal pathway.

Figure 7 The expression of OPN in human endometrium between successful and failed pregnancy group. (A) The protein level of OPN in the endometrium obtained from failed (F) and pregnancy (P) group. (B) The quantification results of OPN protein expression in the endometrium obtained from failed (F, n=10) and pregnancy (P, n=10) group. **P*<0.05.

Successful implantation is dependent on high-quality embryo and the appropriate endometrial receptivity. The primary reason for implantation failure in ART is still an improper uterine function. The accumulation of specific cytokines, growth factors, adhesion molecules, as well as immune cells in endometrium is important for uterine receptivity establishment (Zhang *et al.* 2013). OPN is specifically highly expressed in receptive endometrium in humans and mice (Johnson *et al.* 2003, Qi *et al.* 2014), and functional blockade of OPN significantly inhibited implantation (Zhang *et al.* 2013). Previous study reported that endometrial biopsy could improve the pregnancy rate in IVF patients with recurrent implantation failure, the biopsy process may cause the differentiation of monocyte into dendritic cells, by which the OPN and its receptors were induced in endometrial cells, these processes were regarded as molecular mechanism of biopsy-related successful pregnancy (Gnainsky *et al.* 2015). Our study demonstrated that the OPN is highly expressed in receptive endometrium and decidualized hESC, downregulation of OPN will impede the decidualization process of hESC *in vitro*. Furthermore, the protein level of OPN is significantly repressed in the failed group when compared with successful pregnancy group in IVF/ICSI-ET cycles. Collectively, these evidences strongly suggest that OPN regulates decidualization and vessel remodeling during embryo implantation, aberrant OPN expression in endometrium may lead to implantation failure in humans.

In conclusion, the present results revealed that OPN is involved in endometrial stromal cell decidualization and angiogenesis, which is regulated by cAMP and C/EBPβ. Moreover, we supposed that the downregulation of miR181b may facilitate decidualization via the upregulation of OPN. Besides, clinical trials also should be explored to determine the exact role of OPN in forecasting the success of pregnancy and improving pregnancy rate in IVF/ICSI-ET cycles.

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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