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Original Article

Osteopontin regulates trophoblast proliferation and invasion and associates with spontaneous abortion during early pregnancy

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Abstract: Trophoblast proliferation and invasion are the hall marks of early placental development, defects in trophoblast proliferation and invasion may lead to early pregnancy loss. However, the key molecules that participate in these processes are largely unknown. The study presents here showed that the mRNA and protein levels of osteopontin (OPN) are down-regulated in placental villi tissue from patients with spontaneous abortion when compared with the matched induced abortion group. Knockdown of OPN expression in human choriocarcinoma trophoblast cell line of JEG-3 with OPN shRNA plasmid could attenuate trophoblast proliferation and invasion. PI-3k inhibitor treatment will block the OPN induced P-AKT and MMP-9 expression in JEG-3 cells. In summary, this work describes a direct, positive effect of OPN on the control of human trophoblast invasion by modulation of MMP-9 expression through PI-3k/AKT signal pathway, suggesting that OPN is involved in the pathological process of spontaneous abortion that characterized by insufficient trophoblast invasion.

Keywords: Osteopontin, early pregnancy loss, trophoblast, proliferation, invasion

Introduction

Human reproductive process is characterized by low fertility and high rate of pregnancy loss. It has been reported that approximately 15% of pregnancy ended with early pregnancy loss, in which fetal genetic defects may contribute to 50% of pregnancy loss, while the abnormalities in embryo implantation and placentation have been mentioned as main mechanisms that contributed to early pregnancy loss [1, 2]. The processes of embryo implantation and trophoblast invasion are considered the most limiting factors for the successful of pregnancy [3]. Trophoblast cell proliferates and differentiates into several types of trophoblasts after adhere to endometrium, including villous trophoblasts (VTs) and extravillous trophoblasts (EVTs). Invasive trophoblast derived from proliferative cell columns of anchoring villi, subsequently invade into stroma and arterial vessels of maternal tissues, remodeling of the uterine arteries by these extravillous trophoblasts isresponsible for initiation and maintaining the blood supply of fetal and placenta [4]. The process of invasive trophoblast differentiation is important for placentation and the establishment of fetal-maternal crosstalk.

Osteopontin (OPN) was originally described as a secreted extracellular matrix glycoprotein that participates in cell proliferation and invasion and to contribute to tumorigenesis in several types of cancers [5, 6]. Our previous results explored that OPN is expressed in mouse blastocyst, the glandular epithelium and uterine luminal fluid on day 4 of pregnancy, exerting its role in blastocyst hatching, embryo development and blastocyst adhesion in mouse [7]. It has been suggested that OPN is involved in trophoblast proliferation and invasion during pregnancy, since OPN is strongly expressed in extravillous trophoblast of the villous trophoblast, by which the trophoblast could invade into maternal endometrium and uterine spiral arteries to facilitate placentation and tissue remodeling [8, 9]. These data indicated that OPN may participate in early placentation through involving in trophoblast proliferation and invasion.

As a cytokine, OPN could bind to its cell surface receptors through its arginine-glycine aspartate (RGD)-binding motif, including integrins and CD44, and initiates several kinase pathways that closely related to cell proliferation and invasion [10, 11]. OPN is highlighted as a diagnostic marker for malignant tumors like ovarian cancer or breast cancer due to its profound role in mediating cell invasion and tumor metastasis, there is a correlation of OPN protein expression and pathological stage across a wide variety of tumor histologies, such as carcinomas of the colon, prostate and breast [12-14]. Previous results exposed that OPN treatment could activate focal adhesion kinase and PI-3k/AKT signal pathways in mouse blastocyst, endowing blastocyst with adhesion competence [15]. Therefore, we deduce that the remarkable expression of OPN in trophoblast is associated with placentation by activating trophoblast proliferative and invasive dependent signal pathway.

This work aimed to study the pathological function of OPN in trophoblast proliferation and invasion with special emphasis on whether OPN is involved in spontaneous abortion. We demonstrated that OPN expression is decreased in placental villi tissue from patients with spontaneous abortion, knockdown of OPN in human trophoblastcell of JEG-3 will compromise trophoblast proliferation and invasion. MMP-9, a main protease that participated in trophoblast invasion and tissue remodeling, is regulated by OPN through PI-3k/AKT signal pathway. Our data suggested that OPN is involved in the pathogenesis of spontaneous abortion through regulating trophoblast proliferation and invasion.

Materials and methods

Patients and endometrial sample collection

The study was approved by the Ethics Committee of Renmin Hospital of Wuhan University and all tissue samples were obtained informed consent with the patients before collecting and using. Human chorionic villus tissues were obtained from elective terminations of pregnancy at Renmin Hospital of Wuhan University at the first trimester of spontaneous abortion and induced abortion. There were no significant differences in patient age and pregnancy age between spontaneous abortion pregnancy group and the matched induced abortion group ($P \geq 0.05$). Immediately after curettage, chorionic villus tissues were collected and washed with phosphate-buffered saline (PBS), then part of them were snap frozen in liquid nitrogen immediately and stored at -80°C for RNA and protein assay. The remaining tissue samples were fixed in 10% neutral formalin for 24 h at room temperature, then dehydrated and embedded in paraffin for immunohistochemical staining.

Immunohistochemistry

Formalin-fixed and paraffin-embedded sections were incubated with mouse monoclonal OPN antibody (1:200 dilution, Santa Cruz), CK7 antibody (1:200 dilution, Santa Cruz), or rabbit Ig G (1:200 dilution, Santa Cruz) at 4°C overnight, respectively. After washing in PBS, the sections were incubated with HRP-conjugated secondary antibody (Vector Laboratories, Burlingame, CA, USA) for 45 minutes at room temperature. The color was developed with DAB kit (Vector Laboratories). Positive signal of OPN and CK7 was visualized as brown color. The data demonstrated in each figure was repeated at least three times.

RNA extraction and real-time PCR

Total RNAs from mouse tissues and cultured cells were extracted by Trizol Kit (Sigma) and reverse-transcribed into cDNAs with the PrimeScript reverse transcriptase reagent kit (TaKaRa Bio Inc., Tokyo, Japan). For real-time PCR, cDNA was amplified using a SYBR Premix Ex Taq kit (TaKaRa; DRR041S) on the BIORAD-CFX96™ Real-Time System (BioRad) according to the manufacturer's recommendations. The primer sequences for *OPN* were 5-CACTCCAATCGTCCCTAC-3 and 5-AGACTCACCGCTCTTCAT-3, for GAPDH were 5-AGCCAGAAATCACTGCCACT-3 and 5-TGATCCATGCACCCATAAAA-3. All reactions were run in triplicate. Data from real-time PCR were analyzed by the $2^{-\Delta\Delta Ct}$ method. The significance of differences between the two groups was assessed by Student's t-test. Multiple comparisons were performed with Tukey's ANOVA. $P<0.05$ was considered statistically significant.

Cell lines and culture

The human choriocarcinoma cell line of JEG-3 (ATCC, HTB-36) was purchased from the American Type Culture Collection (ATCC, Rockville, USA) and cultured in Dulbecco's modified

Table 1. Sequences of osteopontin (OPN) short-hairpin RNA (shRNA)

OPN shRNA notation	TagetedOPN mRNA sequence	Loop	Transcription product sequence	Termination signal	Position in GenBank
OPN-homo-1113	GAGCAATGAGCATTCCGATGT	TTCAAGAGA	GAGCAATGAGCATTCCGATGTTTCAAGAGAACATCGGAATGCTCATTGCTCTT	TTTTTTG	1113
OPN-homo-532	GCTGTGTCCTCTGAAGAAACC	TTCAAGAGA	GCTGTGTCCTCTGAAGAAACCTTCAAGAGAGGTTTCTTCAGAGGACACAGCTT	TTTTTTG	532
OPN-homo-356	GAGTTCAATTCCAGTTGAACA	TTCAAGAGA	GAGTTCAATTCCAGTTGAACATTCAAGAGATGTTCAACTGGAATTGAACTCTT	TTTTTTG	356
OPN-homo-406	GGGTCACTGCAATTAGACTGC	TTCAAGAGA	GGGTCACTGCAATTAGACTGCTTCAAGAGAGCAGTCTAATTGCAGTGACCCTT	TTTTTTG	406
OPN-shNC	GTTCTCCGAACGTGTCACGT	TCAAGAGATTA	GTTCTCCGAACGTGTCACGTCAAGAGATTACGTGACACGTTCGGAGAATT	TTTTTTG	None

Eagle's medium (DMEM/F12, hyclone, USA), 10% (v/v) fetal bovine serum (FBS, hyclone, USA), 100 μg/ml penicillin, 100 μg/ml streptomycin (Gibco, USA), maintained at 37°C in a humidified atmosphere of 5% CO_2.

Western blot

Western blots were run as previously reported [16]. Samples were incubated with primary antibodies for OPN (Biorbyt, California, USA), MMP-9 (Cell Signaling Technology, CST, Boston, USA), AKT (CST, Boston, USA), P-AKT (CST, Boston, USA), or β-ACTIN (CST, Boston, USA) and then with matched second antibodies conjugated with horseradish peroxidase. The signals were developed with an ECL chemiluminescent kit (Amersham Biosciences, Boston, USA). All experiments were repeated three times.

Plasmid construction for short hairpin RNA

The full-length sequence of human OPN mRNA was obtained from GeneBank. The short hairpin RNAs (shRNAs) targeting OPN plasmid vectors (pGPU6/GFP/Neo-OPN-homo) and the negative control (shNC) were designed and synthesized by Shanghai GenePharma Company (Shanghai, China) and the vectors containing the neomycin resistant gene (NEO) can encode green fluorescent protein (GFP) as a marker to label transfection. The shRNAs sequence targeting human OPN and OPN-shNC are shown in **Table 1**.

Cell proliferation assays

Cell proliferation assays were performed by cell counting kit (CCK-8 kit, Dojido, Japan). According to the manufacture's instruction, the isolated JEG-3 cells were suspended in DMEM/F12 with 10% FBS and seeded at a density of 8×10^3 cells/well in a 96-well flat-bottom plate. After 24 h of culture, the cells were treated with DMEM/F12 containing 1% FBS for 12 h. The recovered JEG-3 cells were treated with shOPN or shNC for 24 h, 48 h and 72 h, respectively. At the endpoint, 10 μl CCK-8 (5 g/L) was added for further 2 h at room temperature. The plated cells were analyzed immediately using a spectrophotometer microplate reader at the wavelengths of 450 nm. The results are expressed as the ratio of the optical density of cells with treatment to that without treatment. The assays were conducted in triplicate and repeated at least 3 times.

Statistical analysis

Data on cellularity, migration, and invasiveness using a single reagent were analyzed by one-way ANOVA followed by the Tukey multiple comparison test. When two reagents were added in combination, to determine any interactive effect, data were log-transformed and analyzed by two-way or factorial ANOVA followed by a least squares means multiple range test. Because the data were not always normally distributed, the Mann-Whitney rank sum test was employed to determine the level of significance in differences in pairs of various treatment groups. Differences were accepted as significant at $P<0.05$.

Results

OPN expression is decreased in placental villi tissue from patients with spontaneous abortion

It is well recognized that OPN is expressed in human placenta especially the trophoblast cells [17]. Inadequate trophoblast proliferation and invasion is one of the greatest causes of early pregnancy loss and lead to spontaneous abortion [1]. Real-time PCR and western blot were performed to detect the OPN expression infirst-trimester placental villi tissues from patients with spontaneous abortion (SA) and induced abortion (IA), respectively. The results revealed that the mRNA and protein levels of OPN in SA group are both down-regulated when compared with IA group (**Figure 1A** and **1B**). In addition, we observed the localization of OPN protein in villi tissues from patients with SA and IA (**Figure 1C**), as shown in **Figure 1D**, CK7 was used as a positive marker for trophoblast cells, OPN protein is positive expressed in tropoblasts and mainly localized in cytoplasm, the positive signals of OPN in placenta villi tissues from SA group are weaker than IA group.

OPN modulates trophoblast proliferation and invasion in human trophoblast cell of JEG-3

Since OPN expression is suppressed in placenta villi of spontaneous abortion, we supposed that OPN may function in trophoblast proliferation and invasion. Human trophoblast cell of JEG-3 was used to investigate the function of

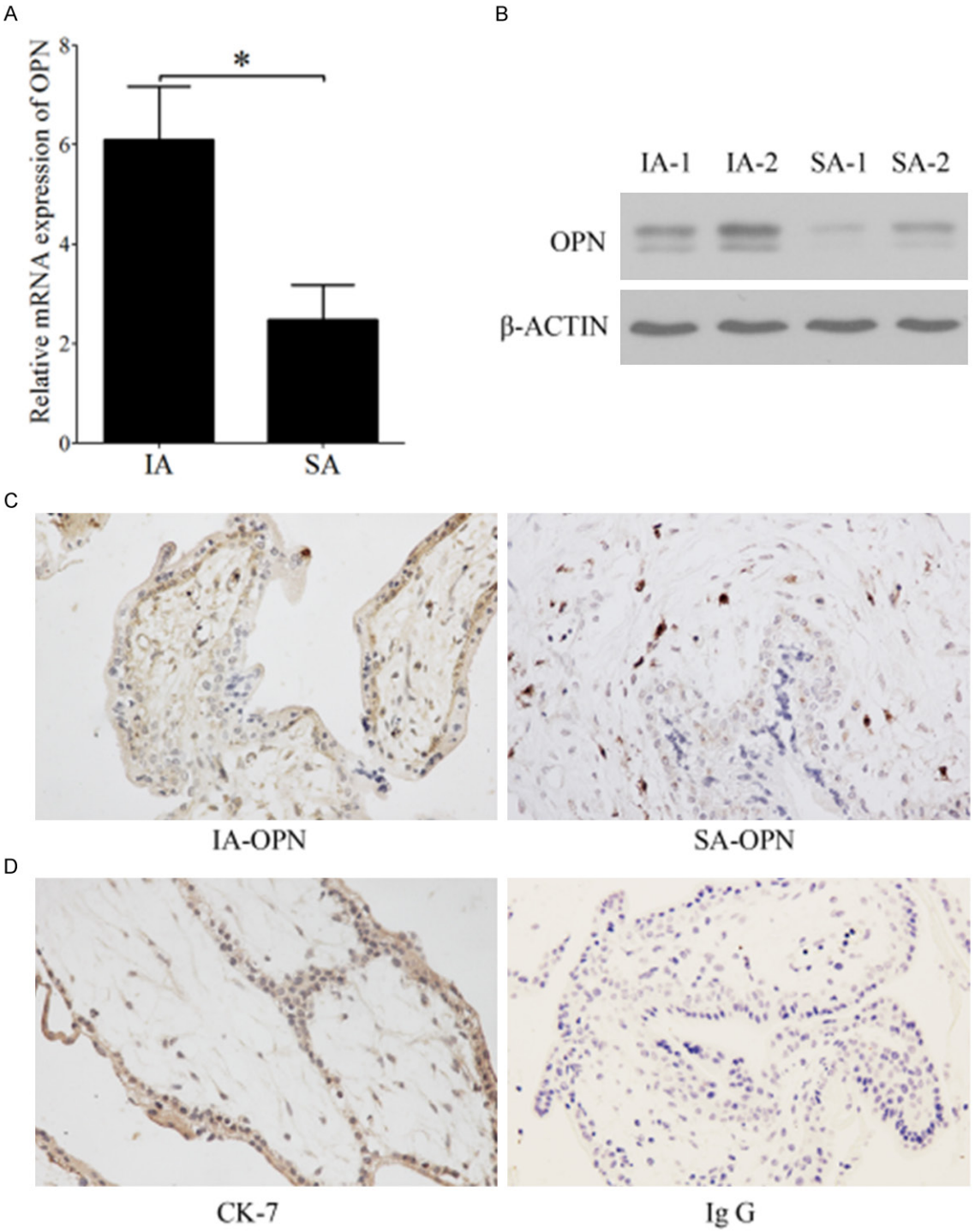

Figure 1. OPN expression in in placental villi tissue from patients with spontaneous abortion or induced abortion. A. Quantitative results of OPN mRNA level in placental villi tissue from patients with spontaneous abortion (SA) or induced abortion (IA). B. Western blot results of OPN protein level in placental villi tissue from patients with SA or IA. C. The localization of OPN protein in placental villi tissue from patients with SA or IA. D. The positive control of CK7 protein localization in placental villi tissue from patients with IA, and the negative control of Ig G in placental villi tissue from patients with IA. $^{*}P<0.05$; *error bars,* S.E. All of the experiments were repeated three times.

OPN in trophoblast proliferation and invasion. JEG-3 cells were transfected with pGPU6/GFP/Neo-OPN-homo plasmid that carried a shRNA targeted to OPN (shOPN). Fluorescence micro-

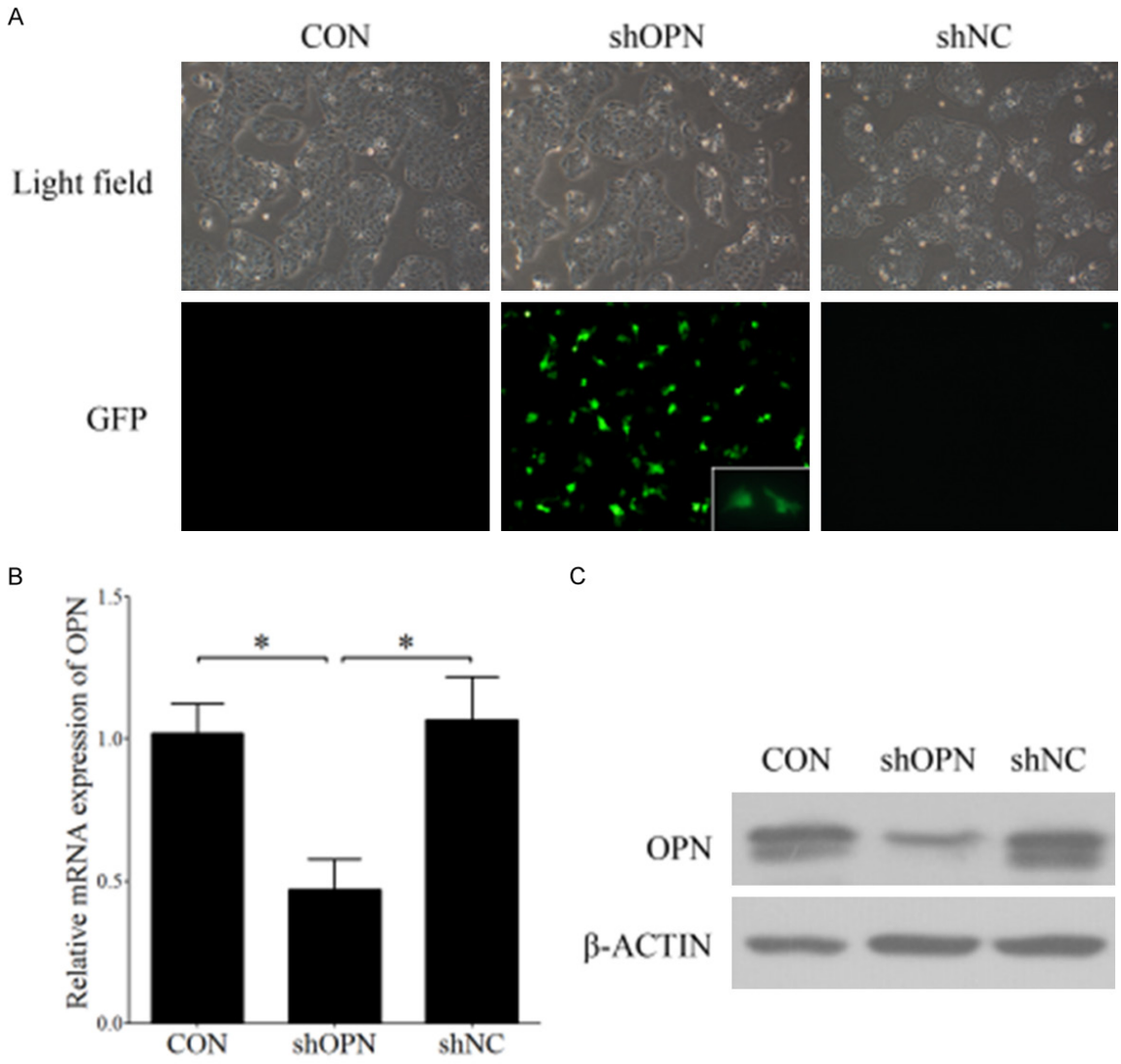

Figure 2. shOPN transfection in JEG-3 cells. A. The light field and GFP immunofluorescence (green) observation of JEG-3 cells treated with blank control, shNC or shOPN, respectively. B. Quantitative results of OPN mRNA level in JEG-3 cells treated with blank control, shNC or shOPN, respectively. C. Western blot results of OPN protein level in JEG-3 cells treated with blank control, shNC or shOPN, respectively. $^{*}P<0.05$; *error bars,* S.E. All of the experiments were repeated three times.

scope was used to verify the transfection efficacy by GFP localization, as shown in **Figure 2A**, the GFP signal was mainly localized in cytoplasm, and the positive rate of GFP localized cells is reached to 70%. In addition, real-time PCR and western blot were used to verify the knockdown efficacy on OPN expression; the results showed the OPN expression is approximately decreased by 60% when compared with control and NC group (**Figure 2B** and **2C**).

Next, we examined the proliferation ability of JEG-3 cells after OPN knockdown by CCK-8 kit. The proliferation rate in shOPN transfected group was inhibited when compared to blank control and NC groups (**Figure 3A**). JEG-3 cells are defined as invasive choriocarcinoma cell lines, scratch assay were used to observe the effect of OPN knockdown on cell migration, as shown in **Figure 3B**, after 48 h of culture, the migration and wound healing of JEG-3 cells were blocked by OPN knockdown. In addition, matrigel invasion assay results found that knockdown of OPN could significantly reduce the invasive ability of JEG-3 cells (**Figure 3C** and **3D**). Taken together, these results suggest that knockdown of OPN may impede the proliferation, migration and invasion of JEG-3 cells.

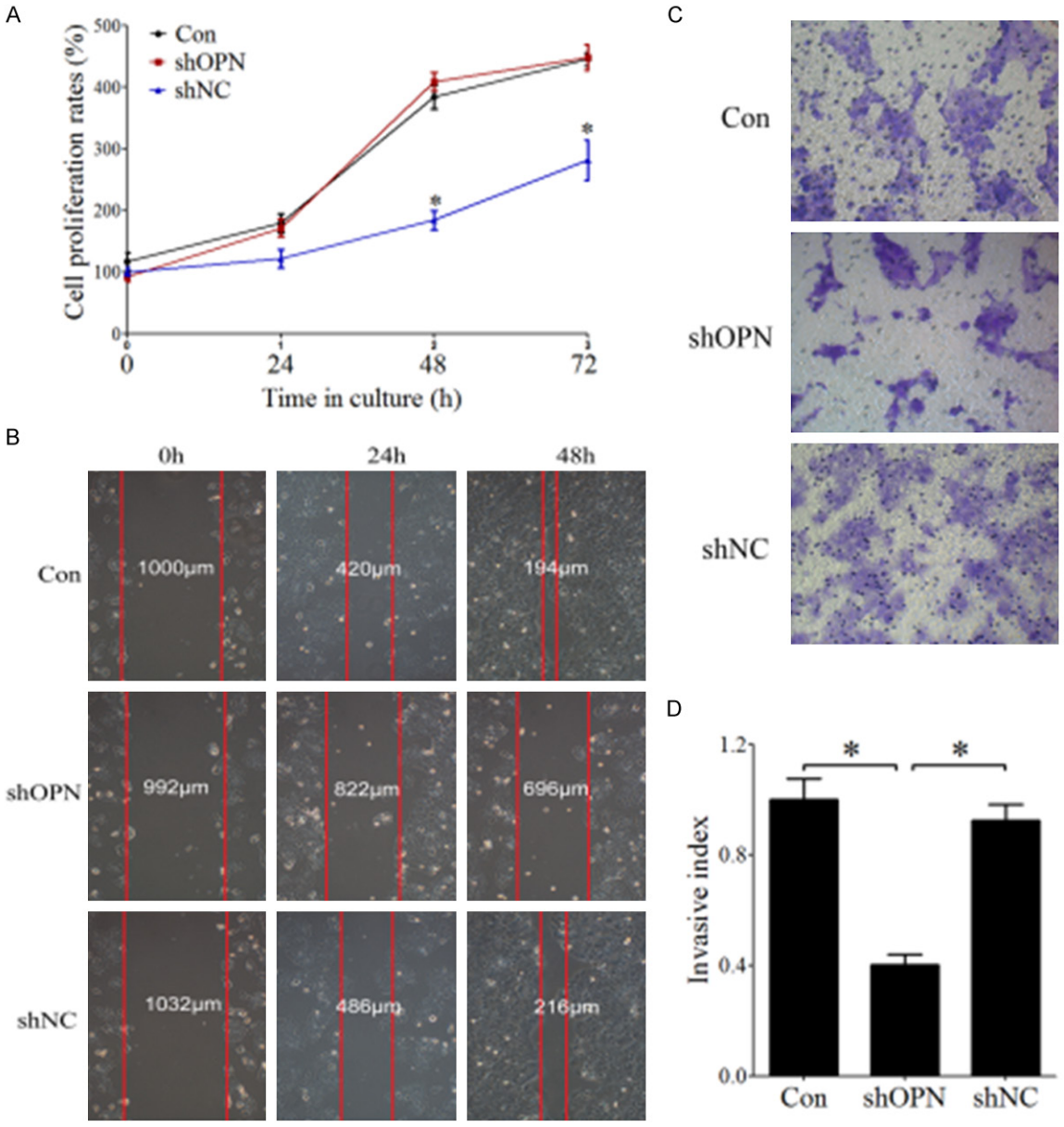

Figure 3. The effect of OPN knockdown on cell proliferation, migration and invasion in JEG-3 cells. A. Cell proliferation rate of JEG-3 cells treated with blank control, shNC or shOPN, respectively. B. Cell scratch and migration assay of JEG-3 cells treated with blank control, shNC or shOPN, respectively. C. Matrigel invasion assay of JEG-3 cells treated with blank control, shNC or shOPN, respectively. D. The quantitative results of matrigel invasion assay of JEG-3 cells treated with blank control, shNC or shOPN, respectively. $^{*}P<0.05$; *error bars,* S.E. All of the experiments were repeated three times.

OPN regulates MMP-9 expression through PI-3k/AKT signal pathway in JEG-3 cells

It is well known that PI-3k/AKT signaling pathway is essential for cell proliferation and differentiation in many physiological and pathological process, including embryo implantation and placentation [18, 19]. We wondered the possible role of PI-3k/AKT signal pathway in OPN mediated trophoblast proliferation and invasion. LY294002 (LY, Cell Signaling), the PI-3k specific inhibitor, was used totreat JEG-3 cells at the concentration of 5, 15 and 50 μM for 12 h, respectively. Western blot results showed that the P-AKT level is decreased by LY treatment in a concentration-dependent manner (**Figure 4A**). Next, LY was used to treat JEG-3 cells at the concentration of 50 μM for 1 h prior

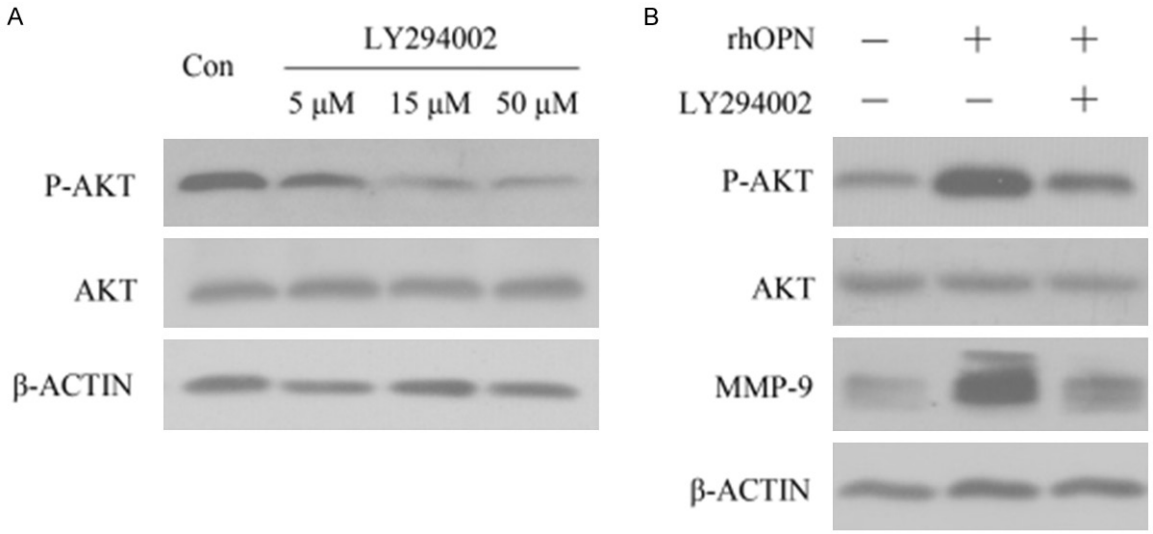

Figure 4. OPN regulates MMP-9 expression through PI-3k/AKT signal pathway in JEG-3 cells. A. P-AKT and AKT protein level in JEG-3 cells treated with specific PI-3k signal inhibitor, LY294002. B. P-AKT, AKT and MMP-9 protein level in JEG-3 cells treated with rhOPN or OPN plus specific PI-3k signal inhibitor, LY294002.

to the treatment of rhOPN, as shown **Figure 4B**, rhOPN treatment promotes the protein level of P-AKT in JEG-3 cells, while LY could suppress the OPN-induced p-AKT expression. Our previous results exposed that OPN is involved in trophoblast invasion through regulating MMP-9 expression [7], in this study, we found rhOPN induced MMP-9 expression in JEG-3 cells is blocked by PI-3k specific inhibitor. In summary, our results illustrate that MMP-9 expression in JEG-3 cells is regulated by OPN through PI-3k/AKT signal pathway.

Discussion

Appropriate trophoblast proliferation and invasion are the prerequisite conditions for the successful of pregnancy, inadequate trophoblast invasion may lead to early pregnancy loss and preeclampsia, while excessive trophoblast invasion is the basis for placenta accrete and trophoblastic diseases [20, 21]. Although a number of studies identified that OPN is distinctively expressed in decidual and placental tissues [22, 23], the specific function and physiological significance of OPN on trophoblast need to be further explored. This study found that OPN expression is significantly decreased in placental villi from the patients with spontaneous abortion, indicating OPN is essential for placenta development, while defect in OPN expression may relate to inadequate trophoblast invasion and lead to early pregnancy loss. In normal placenta, OPN is strongly expressed in the extravillous trophoblast and cytotrophoblast of the villous trophoblast, which are endowed with the abilities of proliferation and invasiveness [17]. In the present study, *in vitro* studies proved that knockdown of OPN will impede the proliferation, invasion and migration processes in JEG-3 cells. In hydatidiform moles, OPN is positivity expressed in the villous cytotrophoblast and proliferative trophoblast on the villous surface, the strongest OPN expression could be observed in the choriocarcinomas, indicating that OPN is possibly involved in promoting trophoblast invasion [9]. However, another results found that OPN is significantly down-regulated in in hydatidiform mole, and in particular complete mole, the role of OPN in hydatidiform mole need to be further verified [24]. Taken together, these results suggest that OPN expression is positive correlated to the ability of proliferation and invasion in trophoblast.

Phosphatidylinositol 3-kinase (PI-3k) pathway regulates several target genes involved in cell survival, proliferation and metabolism [25]. PI-3k/AKT pathway is activated during trophoblast stem cell differentiation, contributing to trophoblast giant cell invasion [26]. Previous results found that OPN promotes mouse blastocyst adhesion via activating AKT signal pathway [15]. Our results showed that protein level of P-AKT is down-regulated by the specific in-

hibitor of PI-3k pathway in JEG-3 cells. rhOPN treatment could activate P-AKT expression in JEG-3 cells, which is blocked by PI-3k inhibitor. MMP-9 is a trophoblast derived MMP, and participates in trophoblast invasion and placentation [27]. rhOPN promoted MMP-9 expression in JEG-3 cells, while the expression of MMP-9 was restrained by adding with PI-3k inhibitor. Previous results found that MMP-9 level is positively correlated to spontaneous early pregnancy failure [28]. Therefore, OPN may regulate MMP-9 expression through PI-3k/AKT in trophoblast cells, and defective expression of OPN and MMP-9 is the possible reason for early pregnancy loss.

Here we present a new insight into the physiological role of OPN during early pregnancy in humans, decreased OPN expression may lead to defects in trophoblast proliferation and invasion, and consequently result in early pregnancy loss. Importantly, we demonstrate that OPN could regulate MMP-9 expression in trophoblast through PI-3k/AKT signal pathway. These results suggest that OPN is important for pregnancy maintaining, and abnormal expression of OPN and MMP-9 is involved in the pathological process of spontaneous abortion.

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Disclosure of conflict of interest

None.

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