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Original Article

Osteopontin facilitates invasion in human trophoblastic cells via promoting matrix metalloproteinase-9 *in vitro*

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Abstract: Successful implantation of embryo and placentation depend on proper trophoblast proliferation and differentiated into specialized invasive trophoblast. However, little is known about the regulatory factors and mechanisms in trophoblast proliferation and differentiation. Osteopontin (OPN) is a member of the small integrin-binding ligand N-linked glycoprotein family and participates in cell adhesion and invasion. It has been identified that OPN is highly expressed in invasive trophoblasts in human placenta. In this study, we demonstrated that OPN is constitutively expressed in highly invasive phenotype of human choriocarcinoma cell lines of JAR and JEG-3 cells, and OPN could promote trophoblast proliferation and invasion, partly through promoting MMP-9 secretion. Inhibition of OPN will compromise the abilities of proliferation and invasion in JAR and JEG-3 cell lines. Our data showed that the expression of OPN in trophoblast may participate in placentation, OPN expression defects may be involved in gestational trophoblastic diseases.

Keywords: Osteopontin, adhesion, trophoblast, invasion

Introduction

In mammals, successful implantation of embryo relies on embryo development, trophoblast invasion, maternal-embryo interaction and immune regulation. The blastocyst adhere to and invade into endometrium through trophoblast invasion, the process of trophoblast invasion bears several striking similarities to tumor cell metastasis, except the trophoblast invasion during normal pregnancy is precisely regulated through the whole process [1]. At the beginning of trophoblast-endometrium interaction, the trophoblast undergoes extensive proliferation under the conditions of hypoxia and the regulations by cytokines and signal pathways [2]. The trophoblast differentiates into several types of trophoblasts and obtain ability of invasion after adhere to endometrium [3]. This precise proliferation and differentiation involves complex and synchronized molecular and cellular events, which are regulated by paracrine and autocrine factors, including cytokines, cell adhesion molecules, hormones, extracellular degrading matrix proteinases and immune cells [4-6].

Osteopontin (OPN) is a secreted extracellular matrix glycoprotein with arginine-glycine aspartate (RGD)-binding motif that combines with αv, β1, β3, and β5 integrin subunits [7]. OPN has been demonstrated to be expressed in several kinds of human tissues, displaying diverse biological functions in cell proliferation, cell adhesion, metastasis and invasion. Our previous results explored that OPN is expressed in mouse blastocyst [8], 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. In addition, OPN is strongly expressed in decidual tissues and immune cells during decidualization, playing a role in maternal-embryo implantation and immune regulation in mouse [7]. Previous results found that remarkable expression of OPN in placenta both in humans and mice. Specifically, OPN is weakly expressed in syncytiotrophoblast that participate in the exchange of nutrient between maternal and fetal systems, while OPN is strongly expressed in extravillous trophoblast and cytotrophoblast of the villous trophoblast, extravillous trophoblast cells of human placenta pro-

gressively lose their proliferative activity and invade into the maternal endometrium and uterine spiral arteries to facilitate placentation and tissue remodeling [9, 10]. This noteworthy expression of OPN in the specialized trophoblast cells may be involved in trophoblast invasion and normal placentation.

The invasion of the trophoblast into stroma depends on the embryo-secreted proteinases, which degrade the extracellular matrix (ECM) components [11]. MMP-9 is the predominant MMP secreted from activated blastocysts, and its expression begins around Day 6, when the blastocysts begin to invade the maternal stroma [12]. Published results suggest that OPN could induce the expression of MMP-9 in some cell types to mediate cell invasion in tumorigenesis [13, 14], our previous results indicated that knockdown of OPN in uterine stromal cells will compromise the expression of MMP-9 in mouse trophoblast with subdued trophoblast outspreading and invasion [7]. Therefore, we deduce that OPN may regulate trophoblast invasion and placentation via up-regulating the expression of MMP-9 in human trophoblasts.

This work aimed to study the expression of OPN in human choriocarcinoma cell lines of JAR and JEG-3 cells with special emphasis on whether OPN is involved in trophoblast proliferation and invasion. We demonstrated that OPN is constitutively expressed in JAR and JEG-3 cell lines, and OPN could promote trophoblast proliferation and invasion, partly through activating MMP-9 secretion, inhibition of OPN will compromise the abilities of proliferation and invasion in JAR and JEG-3 cell lines. Our data showed that the expression of OPN in trophoblast may participate in placentation, OPN expression defect may be involved in gestational trophoblastic diseases.

Materials and methods

Cell lines and culture

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

Immunofluorescence

Formalin-fixed cell plates were incubated with a mouse monoclonal OPN antibody (1:200 dilution, Santa Cruz) or rabbit Ig G (1:200 dilution, Santa Cruz) at 4°C overnight, respectively, followed by the Alexa Fluor 594 conjugated secondary antibody (Jackson ImmunoResearch, 1:200). Nuclei were stained with DAPI (Zhongshan Golden Bridge Bio-technology, Beijing, China). The immunofluorescence signal was visualized by laser confocal microscopy (LEICA, Germany), and the experiments were repeated three times.

Western blot

Western blots were run as previously reported [15]. Samples were incubated with primary antibodies for OPN (Biorbyt, California, USA) or β-actin (Cell Signaling Technology, 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.

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 JAR and JEG-3 cells were resuspended 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 JAR and JEG-3 cells were treated with or without recombinant human OPN (rhOPN, R&D Systems, Minneapolis, MN, USA) protein at concentrations of 0.1 μg/ml, 1.0 μg/ml and 10.0 μg/ml, and were treated with or without neutralizing OPN antibody (R&D Systems, Minneapolis, MN, USA) at concentrations of 0.01 μg/ml, 0.1 μg/ml and 1.0 μg/ml 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.

Matrigel invasion assay

The motility and invasiveness of JAR and JEG-3 cells were evaluated by the Matrigel invasion assay. We used transwell plates (6.5 mm in diameter) (Costar, Cambridge, MA, USA) containing polycarbonate filters with a pore size of 8.0 μm. The upper surface of the filter was coated with 40 μl Matrigel (BD Biosciences, USA) and dried aseptically. Before using, the Matrigel was rehydrated with 100 μl warm DMEM/F12 for 30 min at room temperature. The isolated JAR and JEG-3 cells (1×10^5 in 200 μl DMEM/F12 with 0.1% BSA) were seeded respectively in the upper chamber. The cells were incubated with rhOPN at different concentrations of 0 (control), 0.1 μg/ml, 1.0 μg/ml, 10.0 μg/ml and anti-OPN at the following concentrations of 0 (control), 0.01 μg/ml, 0.1 μg/ml, 1.0 μg/ml. The lower chamber was filled with 600 μl DMEM/F12 with 20% FBS. The cells were allowed to invade for 24 h in 5% CO_2 at 37°C. The attached upper surface cells were removed. The remaining cells on the lower surface were fixed in paraformaldehyde for 10 min at room temperature and stained with crystal violet. The cells that had migrated to the lower surface were counted under a light microscope in five fields at a magnification of ×200. All experiments were done in triplicate and the invasion index was expressed as the percentage of invaded cell number compared with the corresponding control.

MMP-9 detection assays

The JAR and JEG-3 cells were cultured in 96-well plates and incubated with rhOPN (1.0 μg/ml) and anti-OPN (0.1 μg/ml) for up to 72 h respectively. The secreted MMP-9 levels in the supernatants were quantified using the human MMP-9 ELISA kit according to the manufacturer's protocol (RayBiotech, USA). In this study, the minimum detection limits for MMP-9 in the supernatants were 10 ng/L. Briefly, prepared the standard and created the standard dilution for building standard curve. Then samples and biotin conjugate were added to microwell strips. After incubated for 2 h at room temperature, the microwell strips were washed 3 times with wash buffer, and streptavidin-HRP were added to all wells. After incubated for 1 h, microwell strips were washed 3 times followed by adding TMB substrate. After incubated for about 10-30 min, the stop solution was added. The color intensity was measured at 450 nm using a ELISA microplate reader (Perkin Elmer Laboratories, USA). The concentration of protein was determined according to the standard curve and expressed as ng/L of total protein.

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 is constitutively expressed in JAR and JEG-3 cells lines

Previous results proved that OPN protein is strongly expressed in invasive phenotype trophoblast in human placenta, indicating that OPN may relate to trophoblast invasion [16, 17]. Human choriocarcinoma cell lines of JAR and JEG-3 cells, which are manifested as highly invasive trophoblast cells [18, 19], were used to investigate the expression level of OPN in human trophoblasts. Immunofluorescent staining showed that the signal of OPN protein was localized in cytoplasm in both of JAR and JEG-3 cell lines (**Figure 1A**). Western blotting analysis showed that the molecular weight of OPN protein expressed in JAR and JEG-3 cell lines is 45 kDa (**Figure 1B**). The quantitative results indicated that the protein level of OPN in JAR was significantly lower than that of in JEG-3 (**Figure 1C**).

OPN promotes the cell proliferation in JAR and JEG-3 cell lines

Trophoblast proliferation and differentiation are critical events during placentation. OPN was proved to be strongly expressed in the extravillous trophoblast and cytotrophoblast of the vil-

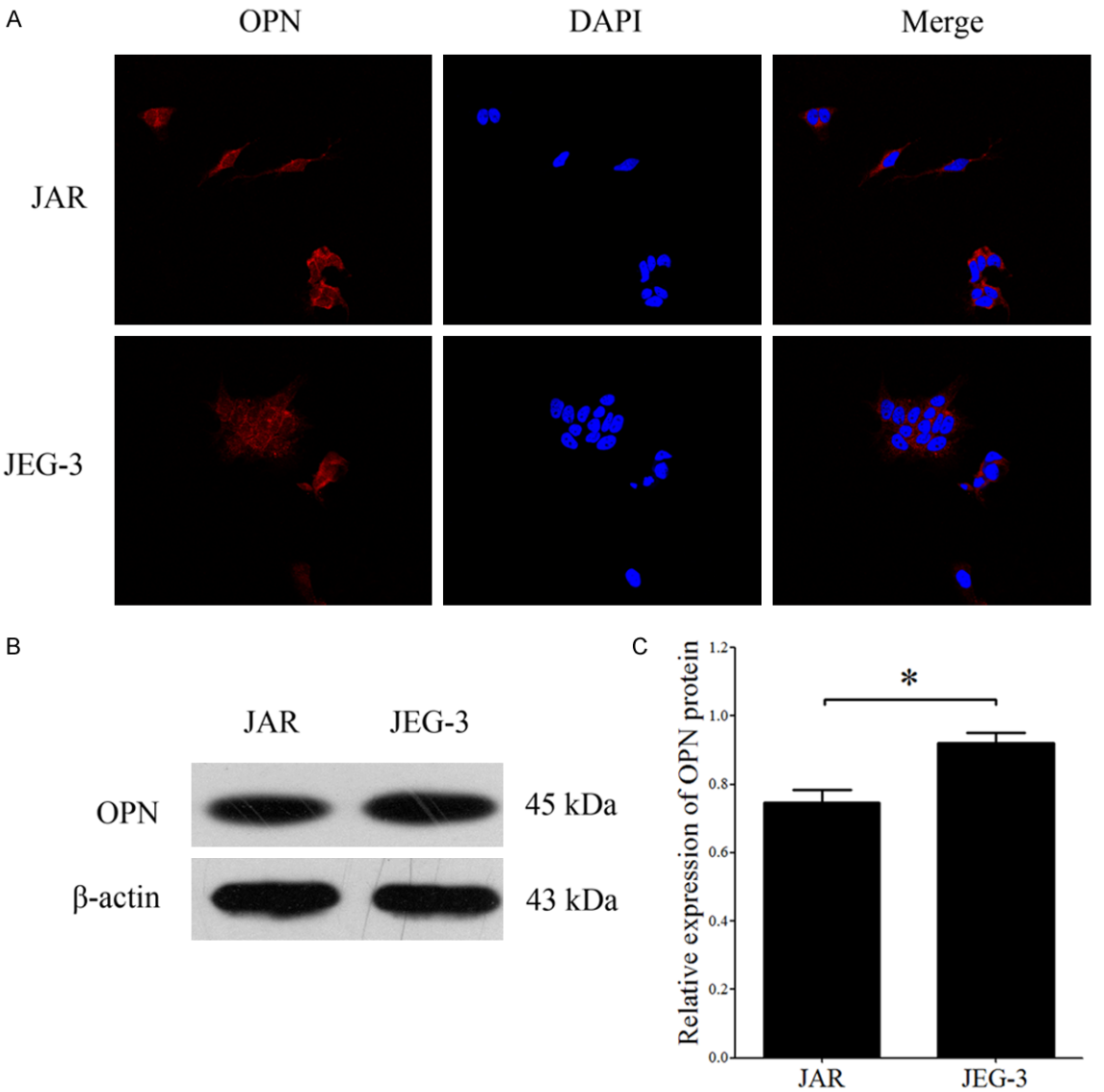

Figure 1. The expression and localization of OPN in JAR and JEG-3 cell lines. A. Representative immunofluorescence staining of OPN in JAR and JEG-3 cells. B. OPN protein level in JAR and JEG-3 cells. C. Quantitative results of OPN protein level in JAR and JEG-3 cells. *P < 0.05; *error bars,* S.E. All of the experiments were repeated three times.

lous trophoblast with the characteristics of highly proliferation and invasion [15]. We deduced OPN may facilitate trophoblast proliferation and involved in placentation. To determine the effect of OPN on trophoblast cell proliferation, JAR and JEG-3 cell lines were treated with recombinant human OPN protein (rhOPN) at the concentration of 0.1, 1.0 and 10.0 μg/ml for up to 72 h culturing, respectively. BSA was used as control. Compared with BSA and 0.1 μg/ml rhOPN treated groups, the proliferation rates of JAR and JEG-3 cells were significantly increased at the concentration of 1.0 and 10.0 μg/ml at 48 and 72 h, respectively (**Figure 2A** and **2B**).

OPN enhances the invasiveness of JAR and JEG-3 cell lines

During the normal placentation, the trophoblast differentiates from a proliferative to an invasive phenotype with the change of uterine environment [20]. JAR and JEG-3 cells are defined as invasive choriocarcinoma cell lines with positive expression of OPN. To determine whether OPN is involved in trophoblast inva-

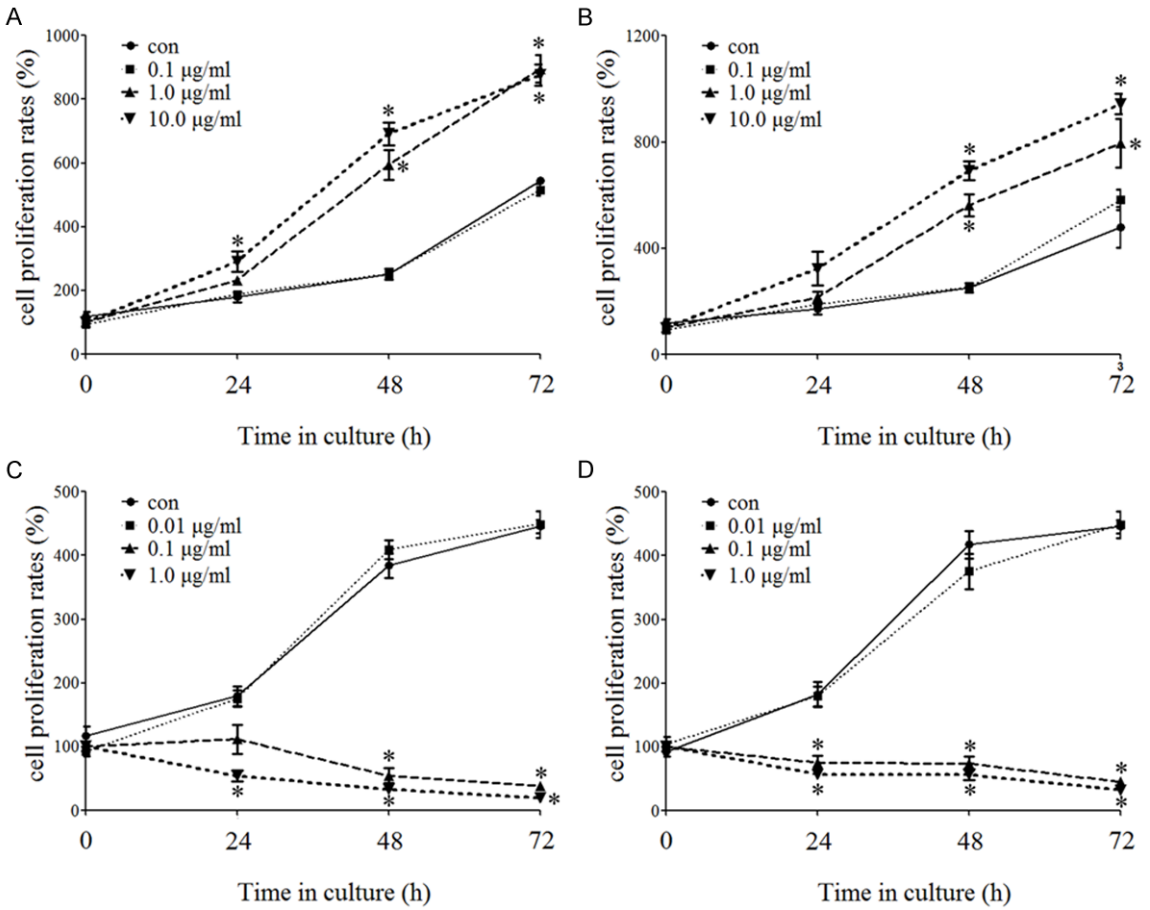

Figure 2. The effect of OPN on cell proliferation of JAR and JEG-3 cells. A. Cell proliferation rate of JAR cells treated with rhOPN at the concentration of 0.1 μg/mL, 1.0 μg/mL or 10.0 μg/mL, respectively. B. Cell proliferation rate of JEG-3 cells treated with rhOPN at the concentration of 0.1 μg/mL, 1.0 μg/mL or 10.0 μg/mL, respectively. C. Cell proliferation rate of JAR cells treated with anti-OPN antibody at the concentration of 0.01 μg/mL, 0.1 μg/mL or 1.0 μg/mL, respectively. D. Cell proliferation rate of JEG-3 cells treated with anti-OPN antibody at the concentration of 0.01 μg/mL, 0.1 μg/mL or 1.0 μg/mL, respectively. $^{*}P < 0.05$ when compared with control group, *error bars,* S.E. All of the experiments were repeated three times.

sion, JAR and JEG-3 cell lines were treated with rhOPN (0.1, 1.0 and 10.0 μg/ml) and anti-OPN neutralizing antibody (0.01, 0.1 and 1.0 μg/ml), respectively. Matrigel invasion assay results showed that supplementation with rhOPN at the concentration of 1.0 and 10.0 μg/ml could prominently enhance the invasion process of JAR and JEG-3 cell lines (**Figure 3A** and **3B**), the quantitative results showed that the invasion index of JAR and JEG-3 cells was significantly enhanced by treating with 1.0 and 10.0 μg/ml rhOPN (**Figure 3C** and **3D**). Accordingly, the matrigel invasion assay and quantitative results demonstrated that the invasion process of JAR and JEG-3 cells was attenuated by treating with anti-OPN neutralizing antibody at the concentration of 0.1 and 1.0 μg/ml (**Figure 4A-D**).

OPN stimulate the secretion of MMP-9 in JAR and JEG-3 cells

During placentation process, trophoblast invasion and uterine decidualization are closely related to tissue reconstruction, including cell proliferation and ECM degradation. MMP-9 is highly expressed in invasive trophoblast, previously results found that OPN could promote cell invasion in tumorigenesis through activating MMP-9 expression. Therefore, we examined the effect of rhOPN (1.0 μg/ml) or anti-OPN (0.1 μg/ml) on MMP-9 production in cultured JAR and JEG-3 cells. As show in **Figure 5A** and **5B**, the secretion of MMP-9 was significantly induced by treating with rhOPN when compared with control in both of JAR and JEG-3 cells after

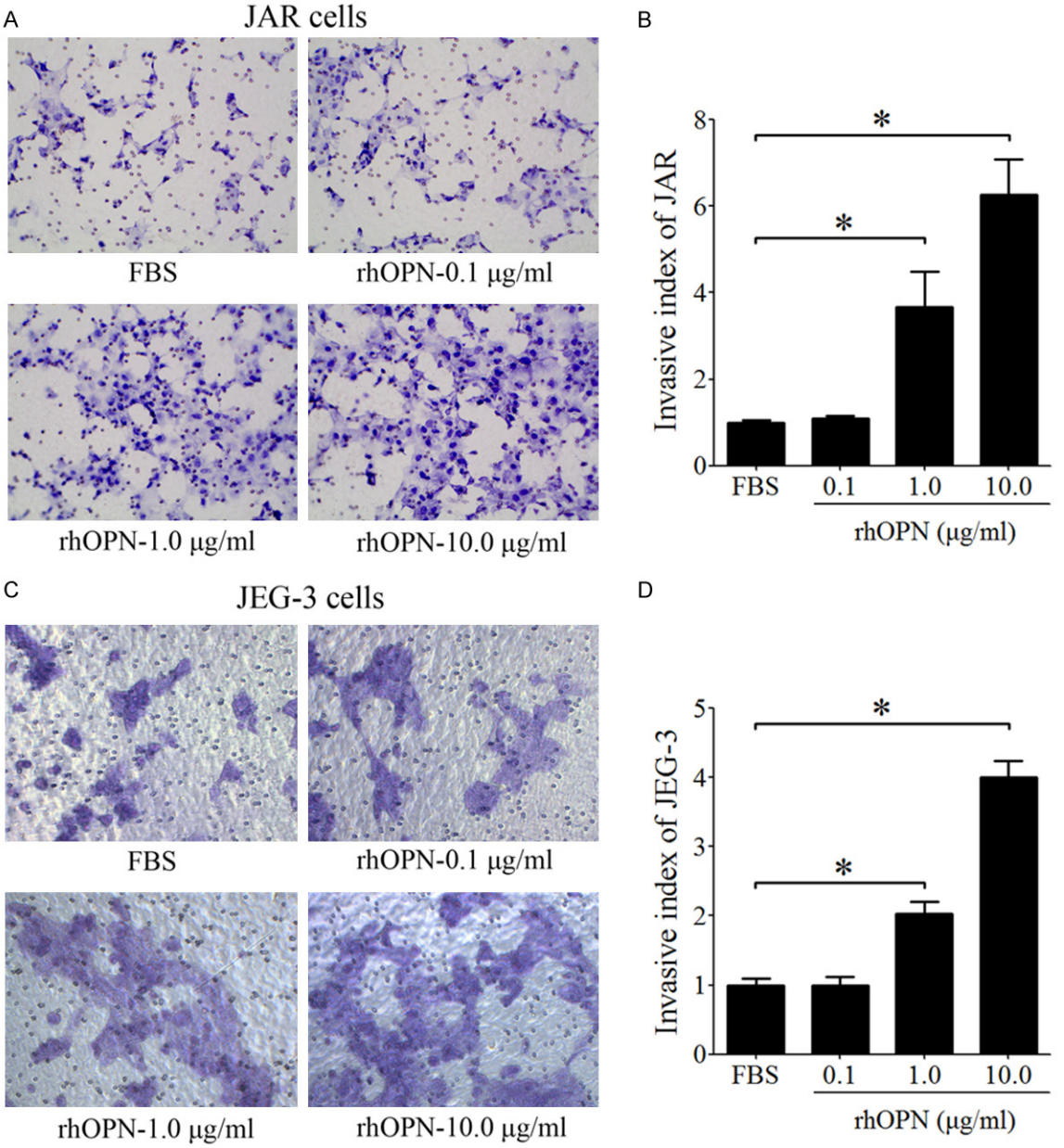

Figure 3. The invasiveness of JAR and JEG-3 cells treated with rhOPN. A. Matrigel invasion assay of JAR cells treated with rhOPN at the concentration of 0.1, 1.0 and 10.0 μg/ml. B. The invasive index of JAR cells treated with rhOPN at the concentration of 0.1, 1.0 and 10.0 μg/ml. C. Matrigel invasion assay of JEG-3 cells treated with rhOPN at the concentration of 0.1, 1.0 and 10.0 μg/ml. D. The invasive index of JEG-3 cells treated with rhOPN at the concentration of 0.1, 1.0 and 10.0 μg/ml.

24, 48 and 72 hours. On the contrast, the amount of MMP-9 was prominently decreased by treating with anti-OPN antibody after 24, 48 and 72 hours (**Figure 5C** and **5D**).

Discussion

It is known that OPN is functionally expressed in several invasive cells, including cancer cells and trophoblast, and OPN plays diverse roles in cell-cell interaction, proliferation, cell adhesion and invasion [7, 16]. In the present study, we showed that OPN is constitutively expressed in human choriocarcinoma cell lines of JAR and JEG-3, which are renowned for their remarkable abilities of proliferation and invasiveness. JAR is a pseudotriploid human choriocarcinoma cell line with the modal chromosome number of 68, while JEG-3 is a hypertriploid human choriocar-

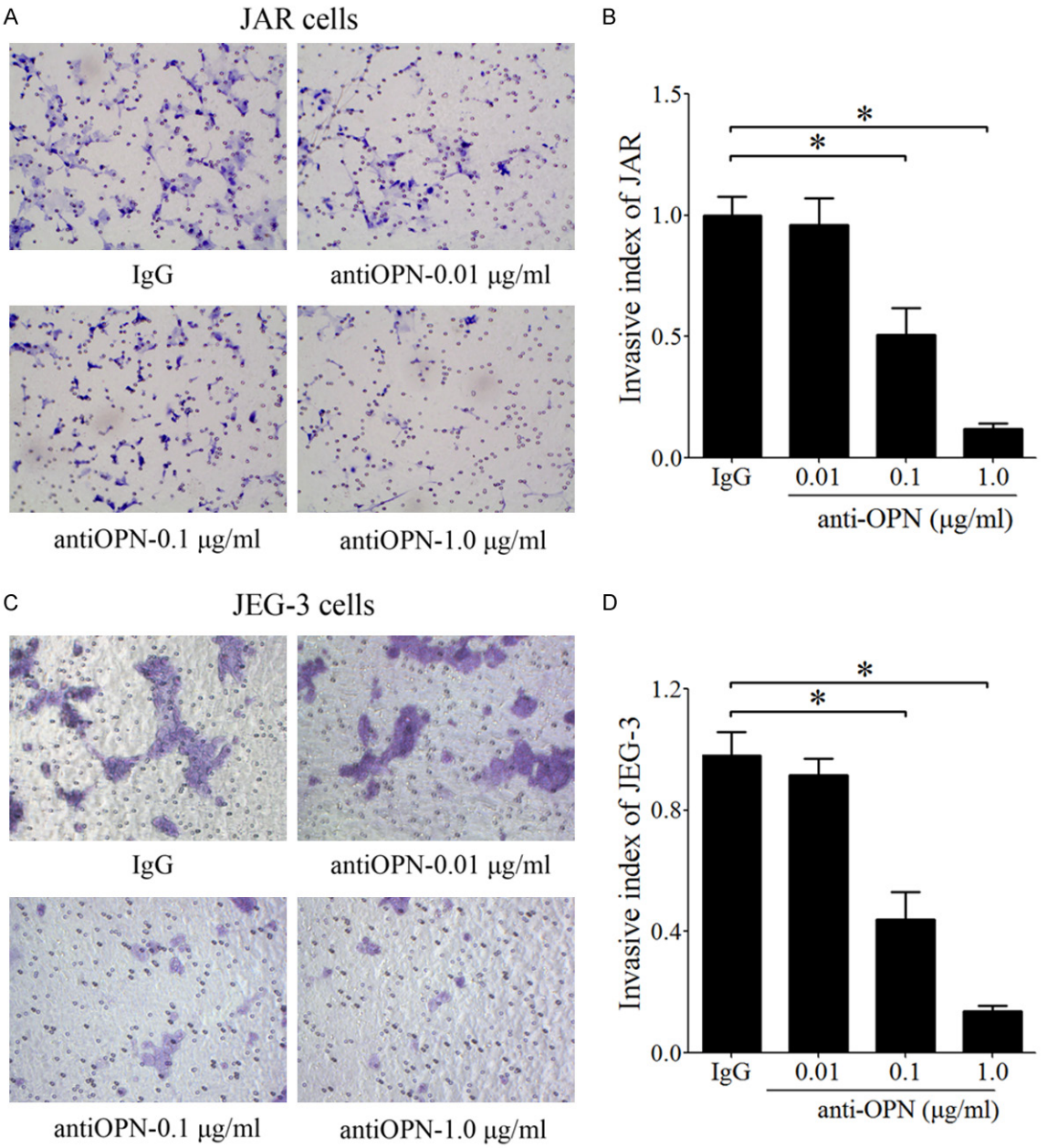

Figure 4. The invasiveness of JAR and JEG-3 cells treated with anti-OPN antibody. A. Matrigel invasion assay of JAR cells treated with anti-OPN antibody at the concentration of 0.01, 0.1 and 1.0 μg/ml. B. The invasive index of JAR cells treated with anti-OPN antibody at the concentration of 0.01, 0.1 and 1.0 μg/ml. C. Matrigel invasion assay of JEG-3 cells treated with anti-OPN antibody at the concentration of 0.01, 0.1 and 1.0 μg/ml. D. The invasive index of JEG-3 cells treated with anti-OPN antibody at the concentration of 0.01, 0.1 and 1.0 μg/ml.

cinoma cell line, in which the modal chromosome number is 71 with more percentage of polyploidy [19], it has been assumed that polyploidy or heteroploid is a marker that highly related to the carcinoma's abilities of invasive or metastasis [21]. The quantitative results of OPN expression in JAR and JEG-3 cell lines showed that OPN is intensively expressed in JEG-3, indicating that high-level expression of OPN in JEG-3 may relate to the high-level of invasiveness.

OPN is a secreted glycoprotein that binds to the corresponding ligand and involved in cell proliferation through activating several proliferative signal pathways, including ERK and AKT path-

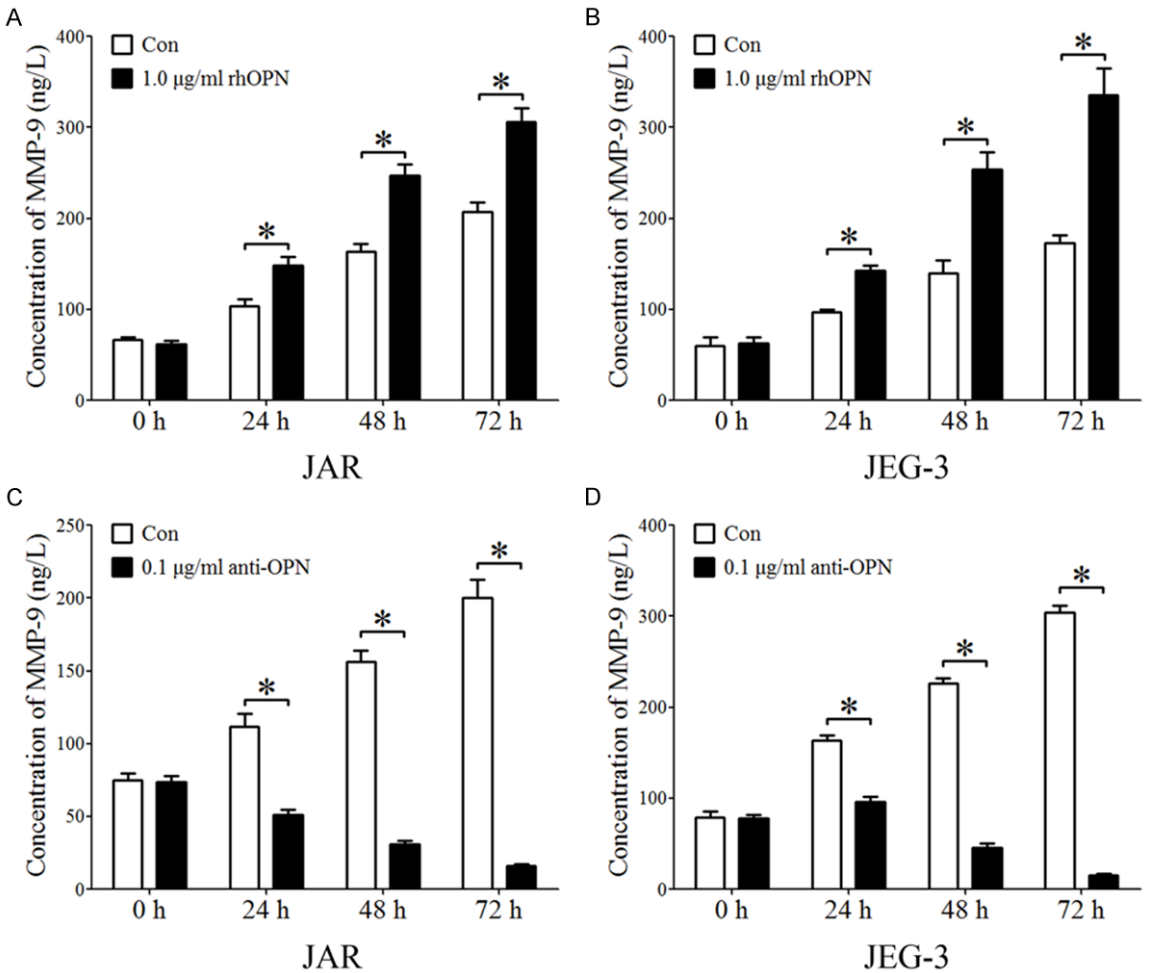

Figure 5. The effect of OPN on MMP-9 secretion in JAR and JEG-3 cells. A. The concentration of MMP-9 in culturing supernatants of JAR cells treated with 1.0 μg/ml of rhOPN for 24, 48 and 72 hours. B. The concentration of MMP-9 in culturing supernatants of JEG-3 cells treated with 1.0 μg/ml of rhOPN for 24, 48 and 72 hours. C. The concentration of MMP-9 in culturing supernatants of JAR cells treated with 0.1 μg/ml of anti-OPN antibody for 24, 48 and 72 hours. D. The concentration of MMP-9 in culturing supernatants of JEG-3 cells treated with 0.1 μg/ml of anti-OPN antibody for 24, 48 and 72 hours.

ways [22]. Trophoblast undergoes extensive proliferation during placentation, our results proved that OPN protein is expressed in highly proliferative human choriocarcinoma cell lines of JAR and JEG-3 cells. In this study, rhOPN supplementation was able to promote the proliferation rate in JAR and JEG-3, while anti-OPN antibody, a neutralizing antibody that binds to secreted OPN, could inhibit the proliferation of JAR and JEG-3. These results indicated that OPN is involved in trophoblast proliferation process, inhibition of OPN may lead to the proliferative defection of trophoblast.

It has been identified that OPN contributes to cell adhesion and invasion, the OPN protein is expressed at high levels in the invading cytotrophoblasts and placenta in humans and mice [9]. Our results showed that supplementation of rhOPN was able to facilitate the invasive process in both of JAR and JEG-3 cells, whereas supplementation of anti-OPN, which is used to neutralize the secreted OPN, could impede the invasive ability of JAR and JEG-3. During placentation, the maternal uterus experience a complex tissue reconstruction process, in which the degrading enzymes secreted from embryo will help trophoblast to invade into uterine endometrium and involve in tissue remodeling [23]. This process depends on the balance of matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs), in which the MMP-9

is identified as trophoblast derived MMP and plays an important role in trophoblast invasion [24]. This present study exposed that supplementation of rhOPN contributed to the augmentation of secreted MMP-9 in both of JAR and JEG-3 cells, while the secretion of MMP-9 was partly blocked by adding with anti-OPN. These results indicated that OPN may encourage trophoblast invasion through facilitating MMP-9 secretion.

The study present here demonstrated that OPN is expressed in human choriocarcinoma cell lines of JAR and JEG-3, OPN expression in JAR and JEG-3 may involve in trophoblast invasion through up-regulating MMP-9, defects in OPN expression may lead to abnormal trophoblast proliferation and invasion. Further studies will be performed to demonstrate the regulating factors and signaling pathways involved in the OPN and normal placentation and the pathogenesis of related placental diseases.

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Disclosure of conflict of interest

None.

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