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Osteopontin Promotes Trophoblast Invasion in the Smooth Muscle Cell-Endothelial Co-Culture At Least Via Targeting Integrin αvβ3

Ru Ke[1,*], Liting Zheng[2,*], Falan Zhao[3], and Junxia Xia[4]

Abstract

Preeclampsia is a pregnancy disorder, whereas the underlying mechanisms and etiological factors of this complication remain elusive. Studies have reported that decreased invasiveness of trophoblast cells, immunity disorder in the maternal–fetal interface, and oxidative stress may contribute to the development of preeclampsia. In the present study, we firstly co-cultured the smooth muscle cells (SMCs) and endothelial cells (ECs) to mimic the decidua and myometrium interface and examined the effects of osteopontin (OPN) on the invasive potential of trophoblasts in the SMC-EC co-culturing system. Our results showed that HTR-8/SVneo cells after hypoxia treatment showed enhanced invasive potential in the SMC-EC co-culturing system. OPN levels in the culture media from hypoxia-treated HTR-8/SVneo cells were significantly increased. More importantly, OPN treatment upregulated integrin, beta 3 and integrin, beta 5 expression in HTR-8/SVneo cells, and promoted HTR-8/SVneo cell invasion in the transwell invasion assay and SMC-EC co-culturing system. Mechanistically, treatment with integrin αvβ3 inhibitor significantly attenuated the enhanced invasive potential of HTR-8/SVneo cells treated with OPN in the SMC-EC co-culturing system. In conclusion, our study for the first time established the SMC-EC co-culturing system to examine the invasive potential of trophoblasts. Our results indicated that OPN promoted the invasive capacity of trophoblasts via at least targeting αvβ3 in the EC-SMC co-culturing system. Future studies were required to further validate the EC-SMC co-culturing system and to determine the molecular mechanisms of OPN-mediated trophoblast invasion.

Keywords

preeclampsia, trophoblast, co-culturing system, osteopontin, integrin αvβ3, invasion

Introduction

Preeclampsia is a pregnant disorder, which exerts a serious impact on the heath of the fetus and perinatal mother[1]. Preeclampsia is featured by the abnormal trophoblast invasion of the uterine spiral arteries and the reduced number of converted vessels[2]. Under normal conditions, human placentation requires the switch of proliferative trophoblasts to an invasive phenotype, and most proliferative trophoblasts within the anchoring and floating villi differentiate to form the syncytiotrophoblasts[3]. The highly invasive trophoblasts will invade through the decidua and myometrium into the maternal spiral arteries[3]. However, in the preeclampsia patients, the normal invasive capacity of trophoblasts was impaired[4]. As far as we know, the precise molecular mechanisms underlying the abnormal invasive behavior of trophoblasts remain elusive.

Osteopontin (OPN) is a class of extracellular matrix glycoprotein and possesses the arginine–glycine aspartate-

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binding motif, which can bind to integrin subunits including αv, β1, β3, and β5[5–7]. OPN has been reported to play diverse biological functions, including cell proliferation, cell invasion, cell adhesion, and so on[8–10]. In the mouse studies, researchers identified the expression of OPN in the mouse blastocyst, the glandular epithelium, and uterine luminal fluid, and OPN plays an important role in blastocyst hatching, blastocyst adhesion, and embryo development in the mouse[11]. OPN was effective in promoting the invasion of human trophoblasts via upregulating matrix metalloproteinase-9[12]. Recently, studies by Frank et al. demonstrated that alpha v integrins could bind OPN to support trophoblast cell adhesion[13]. Previously, our research group demonstrated that OPN and αvβ3 protein were expressed in the human placenta. In addition, OPN was detected in the placental syncytiotrophoblasts and the cytoplasm of capillary endothelial cells, and integrin αvβ3 was mainly expressed on the surface of trophoblast cells[14]. However, the precise mechanisms of OPN and its receptor αvβ3 in the pathogenesis of preeclampsia are not fully understood.

In the present study, we aimed to develop an *in vitro* smooth muscle cell (SMC)-endothelial cell (EC) co-culture system to mimic decidua and myometrium boundary, and further mechanistic studies were performed to examine the effects of OPN on the invasive potential of the trophoblasts in the *in vitro* SMC-EC co-culture system.

Materials and Methods

Cell Lines and Cell Culture

HTR-8/SVneo cells were a generous gift from Professor Fuyuan Qiao (Tongji University, Shanghai, China); human hepatic venous endothelial cells (ED25) were purchased from Cell Bank of Sun Yat-sen University (Guangzhou, China); mouse vascular SMCs (MOVAS-1) were purchased from Type Culture Collection Center of Wuhan University (Wuhan, China). All the three cell lines were cultured in the Dulbecco's modified Eagle medium (DMEM; Gibico, Waltham, MA, USA) supplied with 10% fetal bovine serum (FBS; Gibco) in a humidified incubator at 37 °C with 5% carbon dioxide (CO_2). For generating the green fluorescence protein (GFP)-expressing HTR-8/SVneo cells, a lentiviral vector of pLVX-AcGFP1-C1 (#632155; Takara, Dalian, China) was transfected into 293 T cells with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. At 48 h post-transfection, viral supernatant was collected to infected HTR-8/SVneo cells. The transfection efficiency was evaluated under a fluorescent microscope.

SMC-EC Co-Culture System

The transwell inserts (Costar, Brooklyn, NY, USA) with polycarbonate filters in 8-μm pore size were precoated with 50 μl of 1 mg/ml Matrigel matrix (Becton Dickinson, Bedford, UK). MOVAS-1 cells (0.5×10^5, 1×10^5, 1.5×10^5, and 2×10^5 cells) in serum-free medium were seeded on the lower surface of the inverted transwell inserts. After 2 h culture, the inverted transwell inserts turned upside down and the lower chamber was filled with 2% FBS medium. After a further culture for 24 h, ED25 cells (1×10^5 and 2×10^5 cells) were seeded on the upper chamber and were cultured for another 8 h to establish the SMC-EC co-culture system.

Hypoxia Induction and Drug Treatment

For the hypoxia induction, the HTR-8/SVneo cells or the SMC-EC co-culture were incubated with 250 μM cobalt chloride ($CoCl_2$; Sigma-Aldrich, St. Louis, USA) for 6 h before further *in vitro* assays. For OPN (Sigma-Aldrich) treatment, HTR-8/SVneo cells were incubated with OPN (50 μg/ml) for 6 and 24 h, respectively, before further *in vitro* assays. For the cilengitide (CGT; an integrin αvβ3 inhibitor; Sigma-Aldrich) treatment, HTR-8/SVneo cells were incubated with CGT (20 μg/ml) for 24 h before further *in vitro* assays.

Transwell Invasion Assay Using SMC-EC Co-Culture System

For the invasion assay, GFP-expressing HTR-8/SVneo cells (1×10^5 cells) in serum-free medium were seeded on the upper chamber of the SMC-EC co-culture system. After a further culture for 24, 48, and 72 h, respectively, the cells on the Matrigel side of the inserts were removed by cotton swab, and the invaded cells were examined under a fluorescent microscope.

Transwell Invasion Assay

For the transwell invasion assay, transwell inserts (Costar) with polycarbonate filters in 8-μm pore size were precoated with 50 μl of 1 mg/ml Matrigel matrix (Becton Dickinson). HTR-8/SVneo cells (1×10^5 cells) in serum-free medium were plated in the upper chamber, whereas medium with 10% FBS was added to the lower chamber. After incubating for 24 h, the cells on the Matrigel side of the inserts were removed by the cotton swab. The inserts were fixed in methanol and stained with 0.1% crystal violet (Sigma-Aldrich). The number of invaded cells attached to the lower side of the insert was counted under a light microscope.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA from HTR-8/SVneo cells was extracted using Trizol Reagent (Takara, Dalian, China). RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, USA). qRT-PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems). Amplification and fluorescence detection were performed using an ABI Prism 7500 Real-Time PCR system (Applied Biosystems). Relative integrin subunit beta 3 (ITGB3) and integrin subunit beta 5

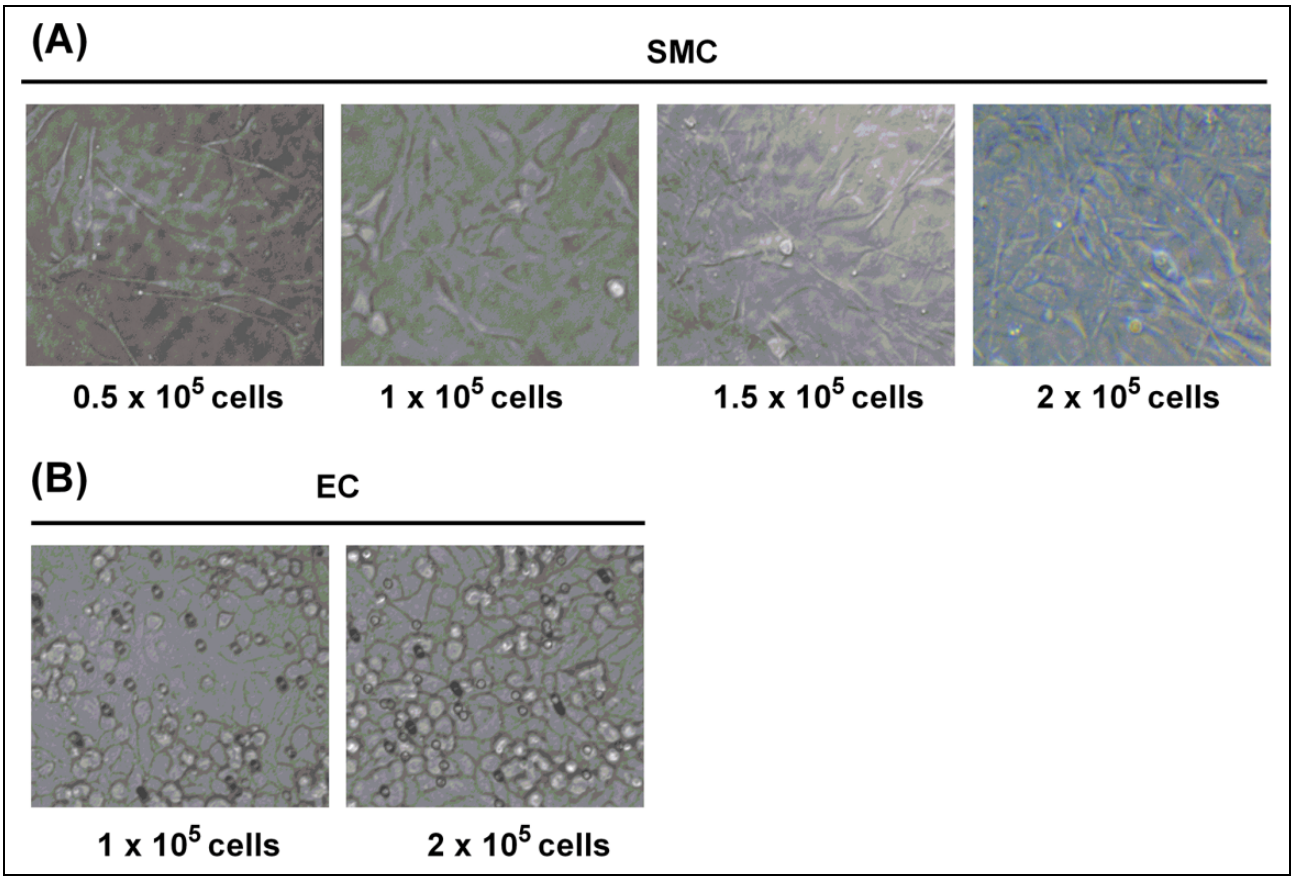

Fig. 1. Optimization of cell seeding number for the SMC-EC co-culture on the transwell membrane. (A) SMC cells (MOVAS-1 cells; 0.5×10^5, 1×10^5, 1.5×10^5, and 2×10^5 cells) seeded on the lower surface of the transwell membrane; the morphology of the SMC cells was evaluated at 24 h after incubating with 2% FBS medium; magnification = 200×. (B) EC cells (ED25 cells; 1×10^5 and 2×10^5 cells) were seeded on the upper transwell membrane followed by SMC cells seeding for 24 h; the morphology of the ECs was evaluated at 8 h after incubating with 2% FBS medium; magnification = 200×. EC: endothelial cell; FBS: fetal bovine serum; MOVAS: mouse vascular SMCs; SMC: smooth muscle cell.

(ITGB5) mRNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the reference control.

ELISA Analysis of OPN Protein Concentrations

The OPN protein concentrations from the culture medium were analyzed using a commercial OPN ELISA kit (#BMS2066; Thermo Fisher Scientific, Waltham, MA, USA) by following the manufacturer's protocol.

Western Blot Analysis

Proteins from cells were extracted using radioimmunoprecipitation assay buffer containing protease inhibitors (Roche, Basel, Switzerland). A total of 50 μg protein samples was resuspended in sodium dodecyl sulfate sample buffer and boiled for 5 min. Equal amounts of protein samples were separated on 10% sodium dodecyl sulfate-polyacrylamide gels and were subsequently transferred to the nitrocellulose membranes. The nitrocellulose membranes were blocked with 5% bovine serum albumin (Sigma-Aldrich) for 1 h at room temperature followed by incubating with primary antibodies against ITGB3 (1:1000; #4702, Cell Signaling Technology, Danvers, USA) and ITGB5 (1:1000; #3629, Cell Signaling Technology), or GAPDH (1:2000; #2118, Cell Signaling Technology). After washing with Tris-buffered saline with 0.1% Tween 20 buffer, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000; Cell Signaling Technology) at room temperature for 2 h. The blotting bands were visualized by enhanced chemiluminescence kit (#32106; Thermo Fisher Scientific). The intensity of the detected bands was quantified by using the Software Smartview 2001 (Shanghai Furi Science & Technology Co. Ltd., Shanghai, China). Relative intensities of each protein signal were obtained by dividing the intensities of each protein signal by those of GAPDH signals.

Statistical Analysis

All the data analysis was performed using the SPSS12.0 software package (IBM, Almond, USA). Unpaired Student's

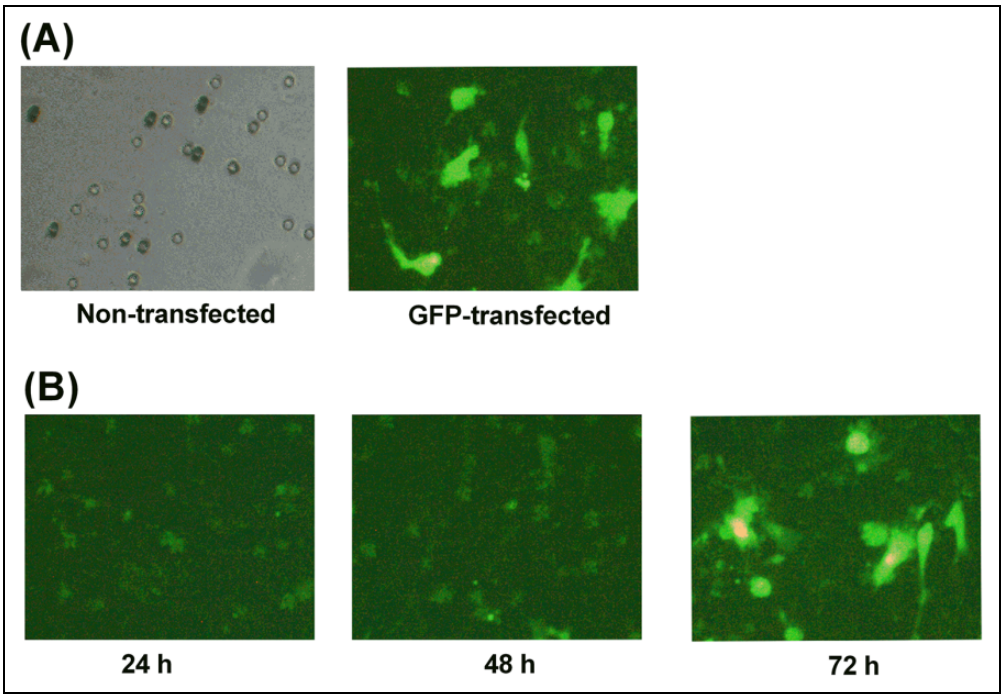

Fig. 2. Optimization of incubation duration for the transwell invasion assay. (A) The GFP expression was achieved by transfecting HTR-8 cells (1.5×10^5) with GFP-overexpressing vector. Left panel shows the HTR-8/SVneo cells without GFP-expressing vector transfection; right panel shows the HTR-8/SVneo cells with the GFP-expressing vector transfection. Images were taken at 24 h after transection; magnification = 200×. (B) The GFP-HTR-8 cells (1.5×10^5) were cultured in the upper chamber of the transwell inserts with SMC-EC co-culture membrane for 24, 48, and 72 h, respectively, and the invaded GFP-HTR-8 cells were assessed under a fluorescent microscope at 24, 48, and 72 h after seeding, respectively. Magnification = 200×. EC: endothelial cell; GFP: green fluorescence protein; SMC: smooth muscle cell.

t-test or one-way analysis of variance followed by Bonferroni's post hoc test was performed to determine the statistical significance between/among different treatment groups, as appropriate. All the data were presented at mean ± SD. The level of statistical significance was set at $P < 0.05$.

Results

Optimization of Cell Seeding Number for the SMC-EC Co-Culture on the Transwell Membrane

In order to establish the SMC-EC co-culture on the transwell membrane, the SMCs were seeded on the lower surface of the transwell membrane at different densities, that is, 0.5×10^5, 1×10^5, 1.5×10^5, and 2×10^5 cells/well, and the cells were cultured for 24 h. As shown in Fig. 1A, SMCs at 1.5×10^5 cells/well cultured for 24 h reached nearly 100% confluence (Fig. 1A). After that, the ECs were seeded on the upper surface of the transwell membrane at the densities of 1×10^5 and 2×10^5 cells/well. After further culturing for 8 h, the ECs at 2×10^5 cells/well showed nearly 100% confluence (Fig. 1B). Based on the above observation, SMCs at 1.5×10^5 cells/well and ECs at 2×10^5 cells/well were used in the subsequent studies.

Optimization of Incubation Duration for the Transwell Invasion Assay in SMC-EC Co-Culture System

In order to optimize the incubation period for HTR-8/SVneo cells, HTR-8/SVneo cells were transfected with vectors expressing GFP. As shown in Fig. 2A, HTR-8/SVneo cells with GFP-expressing vector transfection showed green fluorescence with a transfection efficiency of ~90%, and the transfection of GFP into HTR-8/SVneo cells had no effect on the cell viability (data not shown). Furthermore, we used GFP-HTR-8/SVneo cells to determine the optimized incubating period for cell invasion in the SMC-EC co-culture membrane. The GFP-HTR-8/SVneo cells at 1.5×10^5 cells were seeded on the transwell chamber with SMC-EC co-culture membrane and were cultured for 24, 48, and 72 h, respectively. As shown in Fig. 2B, green fluorescence intensity was relatively low in the lower surface of the transwell when the GFP-HTR-8/SVneo cells were cultured for 24 and 48 h, respectively, while the green fluorescence intensity was markedly increased in the lower surface of the transwell membrane when the GFP-HTR-8/SVneo cells were cultured for 72 h. These results suggested that HTR-8/SVneo cells culturing in the SMC-EC co-culture membrane can be used for the transwell invasion assay.

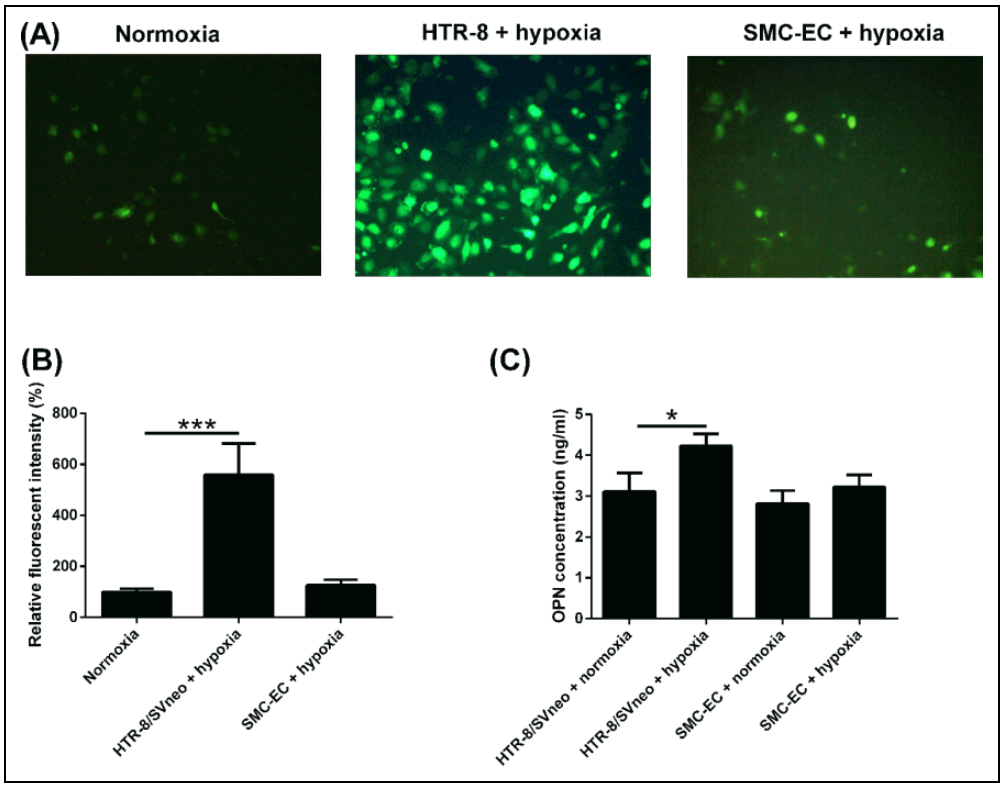

Fig. 3. Effects of hypoxia on the HTR-8 cell invasion in the transwell co-culture system. (A and B) GFP-HTR-8 cells (1.5×10^5 cells) were exposed to hypoxia for 6 h, or SMC-EC co-cultured cells were exposed to hypoxia for 6 h; after that, GFP-HTR-8 cells (were seeded on the SMC-EC co-culture membrane, the invaded GFP-HTR-8 cells were assessed under a fluorescent microscope at 72 h after seeding; magnification = 200×. (C) GFP-HTR-8 cells were exposed to hypoxia for 6 h, or SMC-EC co-cultured cells were exposed to hypoxia for 6 h; after that, GFP-HTR-8 cells (1.5×10^5) were seeded on the SMC-EC co-culture membrane, and the OPN concentration from the culture media was evaluated by ELISA assay at 72 h after seeding. $N = 3$; *$P < 0.05$ and ***$P < 0.001$ indicated the significant differences between different treatment groups. EC: endothelial cell; GFP: green fluorescence protein; OPN: osteopontin; SMC: smooth muscle cell.

Effects of Hypoxia on the HTR-8/SVneo Cell Invasion in the Transwell Co-Culture System

In order to determine the effects of hypoxia on the invasive potential of HTR-8/SVneo cells, we treated the HTR-8/SVneo cells or the SMC-EC co-culture membrane with $CoCl_2$ (250 μM) for 6 h, respectively. GFP-HTR-8/SVneo cells with hypoxia treatment for 6 h showed enhanced invasive potential when compared with the normoxia group (Fig. 3A and B). In addition, SMC-EC co-cultured cells exposed to hypoxia had no effect on the invasive potential of the GFP-HTR-8/SVneo cells when compared with the normoxia group (Fig.3A and B). More importantly, we measured the OPN protein levels in the culture media from hypoxia-treated GFP-HTR-8/SVneo cells and SME-EC co-culture cells, and OPN protein levels in the culture media from hypoxia-treated GFP-HTR-8/SVneo cells but not the SME-EC co-culture cells exhibited higher protein levels of OPN than that in the normoxia group (Fig. 3C). These results suggest that OPN may involve in the invasive potential of HTR-8/SVneo cells induced by hypoxia.

Effects of OPN Treatment on the ITGB3 and ITGB5 Expression in the HTR-8 Cells

Furthermore, we treated the HTR-8/SVneo cells with OPN for 6 and 24 h, respectively, and qRT-PCR and Western blot assays were performed to determine the ITGB3 and ITGB3 mRNA and protein expression levels in HTR-8/SVneo cells. As shown in Fig. 4A, OPN treatment for 6 and 24 h both significantly upregulated ITGB3 and ITGB5 mRNA expression when compared with the control group. Consistently, OPN treatment increased the protein levels of ITGB3 and ITGB5 when compared with the control group (Fig. 4B).

CGT Attenuates OPN-Induced Increase in the Invasive Capacity of HTR-8/SVneo Cells

OPN has been shown to promote the HTR-8/SVneo cell invasion and migration[12], and consistently, we found that OPN treatment significantly promoted the invasion of HTR-8/SVneo cells when compared with the control group (Fig. 5), while treatment with CGT significantly attenuated

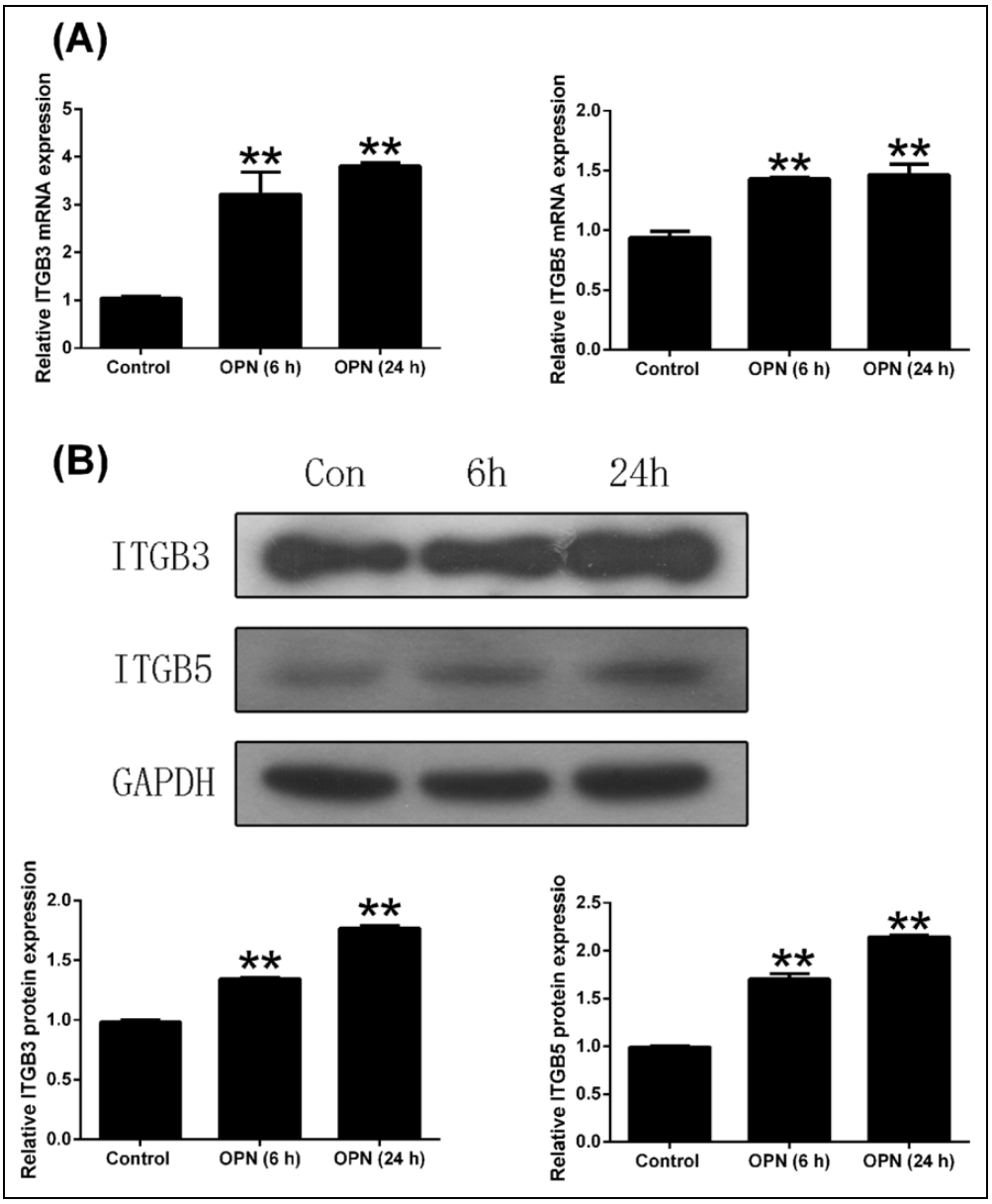

Fig. 4. Effects of OPN treatment on the ITGB3 and ITGB5 expression in the HTR-8 cells. (A) qRT-PCR determination of ITGB3 and ITGB5 mRNA in HTR-8 cells (1×10^5 cells) after being treated with OPN (50 μg/ml) for 6 and 24 h. (B) Western blot analysis of ITGB3 and ITGB5 in HTR-8 cells (1×10^5 cells) after being treated with OPN (50 μg/ml) for 6 and 24 h. $N = 3$; $^{**}P < 0.01$ indicated the significant differences compared with the control group. ITGB: integrin subunit beta; OPN: osteopontin; qRT-PCR: quantitative real-time polymerase chain reaction.

the increased invasive potential of HTR-8/SVneo cells treated with OPN (Fig. 5).

CGT Attenuates OPN-Induced Increase in the Invasive Capacity of HTR-8/SVneo in the Co-Culturing System

Furthermore, we tested the effects of OPN treatment on the invasive potential of GFP-HTR-8/SVneo cells in the co-culturing system. As shown in Fig. 6, OPN treatment significantly promoted the invasion of GFP-HTR-8/SVneo cells in the co-culturing system when compared with the control group (Fig. 6). Expectedly, CGT treatment attenuated the OPN-induced increase in the invasive capacity of HTR-8/SVneo cells in the co-culturing system (Fig. 6).

Discussion

Preeclampsia is a pregnancy disorder, whereas the underlying mechanisms and etiological factors of this complication remain elusive. Studies have reported that decreased invasiveness of trophoblast cells, immunity disorder in the

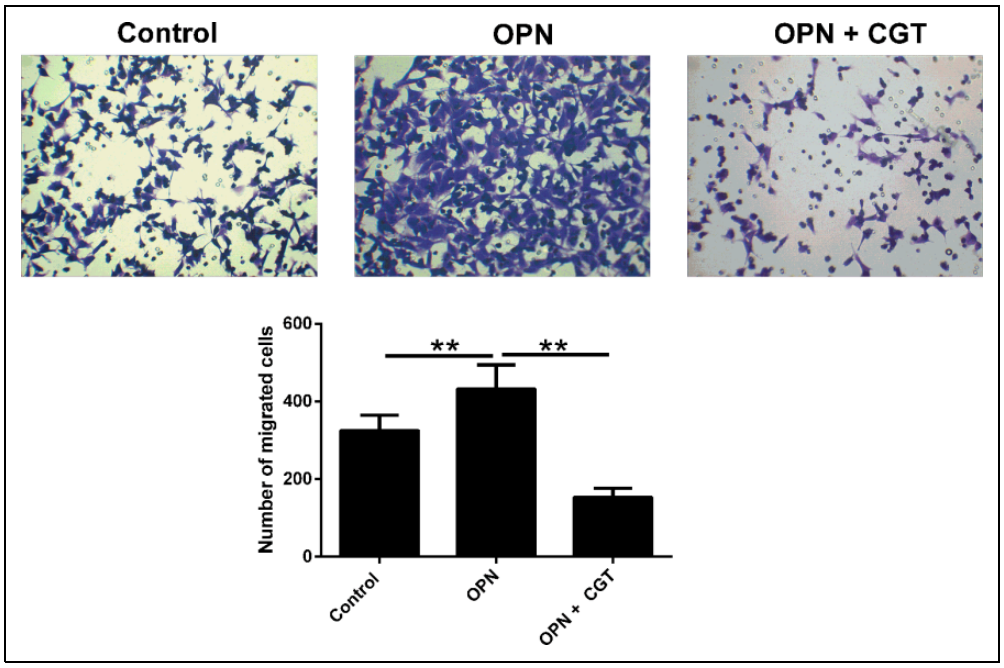

Fig. 5. CGT attenuates the enhanced effects of OPN treatment on the HTR-8 cell invasion. HTR-8 cells (1×10^5 cells) were treated with OPN (50 μg/ml) for 24 h, or the HTR-8 cells (1×10^5 cells) were co-treated with OPN (50 μg/ml) + CGT (20 μg/ml) for 24 h; after that, GFP-HTR-8 cells were seeded on transwell inserts with polycarbonate filters precoated with Matrigel, the number of invaded GFP-HTR-8 cells were assessed at 24 h after seeding. Magnification = 200×. $N = 3$; $^{**}P < 0.01$ indicated the significant differences between different treatment groups. CGT: cilengitide; GFP: green fluorescent protein; OPN: osteopontin;

maternal–fetal interface, and oxidative stress may contribute to preeclampsia development[15–17]. In the present study, we firstly co-cultured the SMCs and ECs to mimic the decidua and myometrium interface and examined the invasive potential of trophoblasts in the SMC-EC co-culturing system. Our results showed that trophoblast cells after hypoxia treatment showed enhanced invasive potential in the SMC-EC co-culturing system. In addition, hypoxia treatment significantly increased the OPN levels in the culture media from HTR-8/SVneo cells. More importantly, OPN treatment upregulated ITGB3 and ITGB5 expression in HTR-8/SVneo cells and promoted HTR-8/SVneo cell invasion in the transwell invasion system and SMC-EC co-culturing system. Mechanistically, treatment with integrin αvβ3 inhibitor significantly attenuated the enhanced invasive potential of HTR-8/SVneo cells treated with OPN in the SMC-EC co-culturing system. Collectively, these results suggested the potential role of OPN and integrin αvβ3 in the pathophysiology of preeclampsia.

The SMC-EC co-culturing systems have been recently applied in the investigation of cardiovascular diseases[18,19]. Zheng et al. found that the phenotype of vascular SMCs co-cultured with endothelial cells is modulated by platelet-derived growth factor receptor beta/IQ motif containing GTPase activating protein 1 signaling in lipopolysaccharides-induced intravascular injury[20]. In the aspect of preeclampsia studies, Dunk et al. developed an *in vitro* co-culture system in which first trimester villous explants are cultured at low oxygen tension in contact with 2-mm^2 sections of decidua parietalis from the same patient[21]. Alison et al. cultured endothelial cells and vascular SMCs in hanging droplets to form spheroids representing an inverted vessel lumen and found that trophoblast-induced changes in C-X-C Motif chemokine 10 expression contributed to vascular SMC dedifferentiation during spiral artery remodeling[22]. In our study, the ECs and SMCs were co-cultured in the upper and lower surface of the transwell, respectively, to mimic the decidua and myometrium interface, and the transwell invasion results showed that HTR-8/SVneo cells required at least 72 h to invade through the co-cultured layers, while in most of the transwell invasion assays, 24 h incubation period is sufficient for the HTR-8/SVneo cells invading through the Matrigel-coated membranes. The collective evidence suggested that the co-cultured layers have an impact on the invasive capacity of the HTR-8/SVneo cells. In early pregnancy, hypoxia is the key factor that affects trophoblast proliferation, invasion, and migration. Trophoblasts treated with 24 h hypoxia showed an enhanced invasion and migration[23]. In our study, we showed that treating trophoblast with 6 h hypoxia did not affect the cell proliferation (data not shown), but induced an increase in the invasive capacity of trophoblasts; while treating SMC-EC co-culturing system had no effect on the trophoblast invasion, suggesting that inducting trophoblasts with hypoxia promotes trophoblast invasion in the SMC-EC co-culturing system.

OPN is a class of extracellular matrix glycoprotein and has been reported to play an important role in regulating the

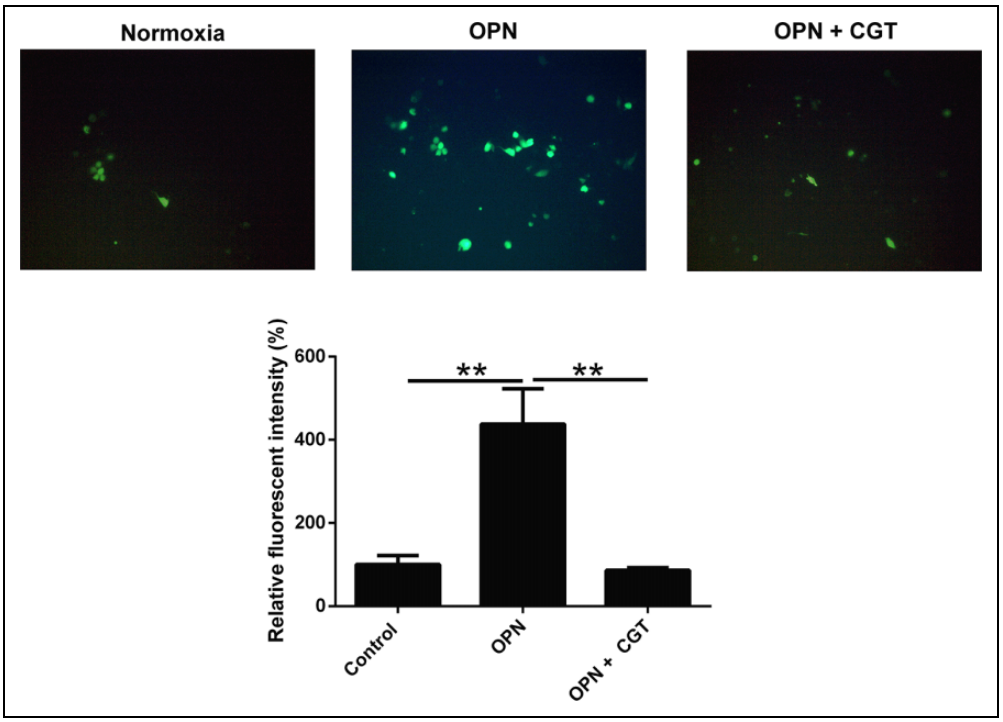

Fig. 6. CGT attenuates the enhanced effects of OPN treatment on the HTR-8 cell invasion in the SMC-EC co-culturing system. HTR-8 cells (1×10^5 cells) were treated with OPN for 24 h, or the HTR-8 cells (1×10^5 cells) were first co-treated with OPN + CGT for 24 h; after that, GFP-HTR-8 cells were seeded on the SMC-EC co-culture membrane, and the cell invasive capacity of HTR-8 cells was evaluated under a fluorescent microscope at 72 h after seeding. Magnification = 200×. $N = 3$; $^{**}P < 0.01$ indicated the significant differences between different treatment groups. EC: endothelial cell; CGT: cilengitide; GFP: green fluorescence protein; OPN: osteopontin; SMC: smooth muscle cell.

invasiveness of trophoblast cells[24,25]. In this study, we showed that OPN protein levels were increased in the culture media from the hypoxia-treated trophoblasts, but not in the hypoxia-treated SMC-EC co-culture, suggesting that increased OPN protein levels may be related to the enhanced invasive activity of trophoblasts. In the subsequent studies, we confirmed that OPN treatment could increase the invasive capacity of trophoblasts in the transwell invasion assay and the SMC-EC co-culturing system. In order to further elucidate the OPN-mediated trophoblast invasion, we performed the qRT-PCR and Western blot analysis and found that OPN treatment upregulated ITGB3 and ITGB5 expression in the trophoblasts. As ITGB3 is a key component of αvβ3 and αvβ3 is expressed on the surface of trophoblast cells[26–28], we speculated that αvβ3 may involve in OPN-mediated trophoblast invasion. OPN treatment for 6 h did not affect the cell proliferative potential of the trophoblasts in our study (data not shown). Furthermore, we found that pretreatment with αvβ3 inhibitor significantly attenuated the OPN-induced increase in the invasive capacity of trophoblasts in the transwell invasion assay and the SMC-EC co-culturing system. Collectively, these results may imply that OPN increased trophoblast invasion at least via targeting αvβ3.

Our findings are still at the preliminary stage and have several limitations. First, the current investigation is limited to the *in vitro* culturing studies to mimic the decidua–myometrium interface, and future studies need to characterize this model as representative *in vivo* situation in the human. Second, our study used the MOVAS line and human hepatic venous ECs, and future studies may consider to use the human primary ECs as well as vascular SMCs to further confirm our findings. Third, the effects of OPN on the cell apoptosis and oxidative stress of the HTR-8/SVneo are unknown, which still requires further studies to confirm the mechanistic actions of OPN on the trophoblasts by using the SMC-EC co-culturing system.

In conclusion, our study, for the first time, established the SMC-EC co-culturing system to examine the invasive potential of trophoblast. Our results indicated that OPN promoted the invasive capacity of trophoblasts via at least targeting αvβ3 in the EC-SMC co-culturing system. Future studies were required to further validate the EC-SMC co-culturing system and to determine the molecular mechanisms of OPN-mediated trophoblast invasion.

Authors' Contributions

JX conceived the study. RK and LZ designed the experiments and wrote the manuscript. RK and FZ analyzed the data and revised the manuscript.

Ethical Approval

This study was approved by our institutional review board.

Statement of Human and Animal Rights

This article does not contain any studies with human or animal subjects.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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