



Effects of Oncostatin M on Invasion of Primary Trophoblasts under Normoxia and Hypoxia Conditions

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Purpose: To investigate the effect of oncostatin M (OSM) on protein expression levels and enzymatic activities of matrix metalloproteinase (MMP)-2 and MMP-9 in primary trophoblasts and the invasiveness thereof under normoxia and hypoxia conditions.

Materials and Methods: Protein expression levels and enzymatic activities of MMP-2 and MMP-9 in primary trophoblasts under normoxia and hypoxia conditions were examined by Western blot and zymography, respectively. Effects of exogenous OSM on the *in vitro* invasion activity of trophoblasts according to oxygen concentration were also determined. Signal transducer and activator of transcription 3 (STAT3) siRNA was used to determine whether STAT3 activation in primary trophoblasts was involved in the effect of OSM.

Results: OSM enhanced protein expression levels and enzymatic activities of MMP-2 and MMP-9 in term trophoblasts under hypoxia condition, compared to normoxia control ($p < 0.05$). OSM-induced MMP-2 and MMP-9 enzymatic activities were significantly suppressed by STAT3 siRNA silencing under normoxia and hypoxia conditions ($p < 0.05$). Hypoxia alone or OSM alone did not significantly increase the invasiveness of term trophoblasts. However, the invasion activity of term trophoblasts was significantly increased by OSM under hypoxia, compared to that without OSM treatment under normoxia.

Conclusion: OSM might be involved in the invasiveness of extravillous trophoblasts under hypoxia conditions via increasing MMP-2 and MMP-9 enzymatic activities through STAT3 signaling. Increased MMP-9 activity by OSM seems to be more important in primary trophoblasts.

Key Words: Oncostatin M, trophoblast, signal transducer and activator of transcription 3, hypoxia

INTRODUCTION

In early pregnancy, the trophoblast differentiates to villous and extravillous trophoblasts (EVT) after implantation.¹ The villous trophoblast gives rise to chorionic villi. EVT then migrates into

the decidua and myometrium and invade maternal vasculature.² The invasion of EVT plays a critical role in the development of fetal-maternal interface. After EVTs invade the decidua and myometrium, they can remodel uterine spiral arteries and change dilated and inelastic tubes without maternal vasomotor control, thus reducing uteroplacental vascular resistance.³ Impaired remodeling causes high uteroplacental resistance and leads to pregnancy complications, such as fetal growth restriction (FGR) and preeclampsia.⁴

Trophoblast invasion in early pregnancy occurs under low oxygen conditions. Hypoxia signaling is considered to be an important regulator of trophoblast invasion.⁵ Low oxygen tension can stimulate vascular development, tissue nutrition, and growth. It also promotes cellular specialization and contributes to placental morphogenesis.⁶ Although the mechanism involved in the effect of hypoxia on EVT invasion has not

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been clarified yet, hypoxia is considered as an important regulator for EVT invasion and placental morphogenesis.⁷

Invasive trophoblasts secrete numerous proteolytic enzymes to degrade basement membranes. Matrix metalloproteinases (MMPs) are zinc-dependent proteolytic enzymes capable of degrading the extracellular matrix. They are thought to be key enzymes in trophoblast invasion.⁸ The invasion of trophoblasts is influenced by various cytokines, including leukemia inhibitory factor,⁹ interleukin-6 (IL-6), hepatocyte growth factor, and granulocyte macrophage-colony stimulating factor.¹

Oncostatin M (OSM), a cytokine of the IL-6 family, can either promote or inhibit cell growth in various cell types. It has been shown to exert various effects on the expression of MMPs according to cell types.¹⁰ OSM is produced by decidual glands and stromal cells during pregnancy. It is related to placental endocrine function.¹¹ Our previous studies have shown that OSM can stimulate the invasion activity of the immortalized human trophoblast cell line HTR8/SVneo through increasing enzyme activities of MMP-2 under normoxia condition.¹² In addition, signal transducer and activator of transcription 3 (STAT3) activation appears to be critical for OSM-mediated invasiveness of HTR8/SVneo cells.^{13,14} The aim of this study was to investigate the effect of OSM on protein expression levels and enzymatic activities of MMP-2 and MMP-9 and invasiveness in human primary trophoblasts under hypoxia and normoxia conditions.

MATERIALS AND METHODS

Isolation of extravillous trophoblast cells from term human placenta

Term placenta tissues from elective cesarean section were obtained immediately after cesarean section with informed consent. FGR, pregnancy-induced hypertension, and placenta previa were excluded from the study. EVTs were isolated by the method described previously with some modification.¹⁵⁻¹⁷ Briefly, the placental tissue was washed in calcium and magnesium free Hank's Balanced Salt Solution (HBSS). The tissues were dissected, rinsed, transferred to a petri dish, and minced to remove blood vessels and calcifications. The chorionic villi in HBSS were digested with trypsin (15 mL), DNase (1.5 mL) in HBSS for 15-20 minutes at 37°C. After sequential digestion, the supernatant from digestion was slowly layered over fetal bovine serum (FBS). We repeated this process three times. Suspended cells were filtered through a 100 µm nylon strainer. The cells were then centrifuged at 622×g for 10 min, after which we removed the supernatant and suspend pellet in 6 mL of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12). Then the Percoll gradients were centrifuged at spin 1200×g for 20 min. We collected cells in the band between 30% and 60% Percoll. After spinning for 10 min at 622×g, the supernatant was aspirated, and the cell pellet

was resuspended in cell culture media.

Cell culture

Isolated EVTs were seeded onto a plate coated with growth factor reduced Matrigel and cultured for 3 days in DMEM/F12 (Sigma D5671, Sigma, St. Louis, MO, USA/Gibco, Carlsbad, CA, USA) medium containing 10% FBS and 0.5 mL gentamicin (Sigma G1397). They were centrifuged at 200×g for 10 min and resuspended in culture media. Cells were cultured for 24 h in a standard 5% CO₂ incubator at 37°C. Cell culture supernatants were harvested and stored at -80°C until analysis. Cells from half of the plates were transferred to an anaerobic chamber (Forma Scientific, Marietta, OH, USA) with an atmosphere of 10% CO₂ and 1% oxygen (designated herein as hypoxic condition). An oxygen monitor in the incubator indicated that the level of oxygen was maintained at 1% to 2% during culture. PO₂ level was ≤14 mm Hg. Cells from the other half of the plates were cultured in 95% air (i.e., 20% oxygen; PO₂ level of 120 to 130 mm Hg) and 10% CO₂ in a humidified atmosphere at 37°C (designated as normoxic condition). To analyze time-dependent effects of OSM, cells were treated with OSM (20 ng/mL, Sigma-Aldrich, St. Louis, MO, USA) and incubated for 12 and 24 h. The EVT was identified by HLA-G immunohistochemistry stain [ImmPRESS™ Excel Amplified HRP Polymer Staining kit (anti-mouse IgG), Vector Laboratories (Burlingame, CA, USA), MP-7602] (Fig. 1).

Protein expression of MMP-2 and MMP-9

Western blot analysis was performed to determine protein expression levels of MMP-2 and MMP-9 as described in our previous study.¹³ The protein was weighted by 40 µg per well. The experiment was performed under normoxic and hypoxic conditions as defined above. Each experiment was replicated three times. Western blot bands were quantified by a Gel Doc™ XR system with Image lab software (Bio-Rad, Hercules, CA,

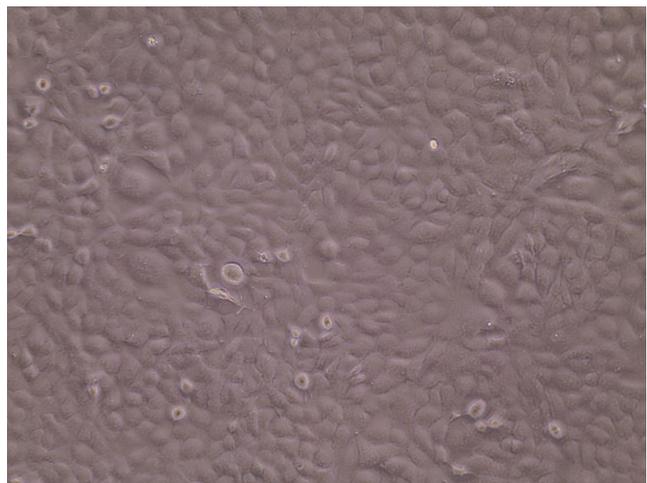


Fig. 1. Primary term trophoblasts after isolation of extravillous trophoblasts (HLA-G immunohistochemistry stain, ×200).

USA). GAPDH was used as a loading control.¹⁸

Effect of OSM treatment on MMP-2 and MMP-9 protein expression levels under normoxia and hypoxia conditions

Primary term trophoblasts were incubated for 24 h and treated with or without OSM (20 ng of OSM at 20 ng/mL) for 12 h and 24 h under the normoxia or hypoxic condition. Western blot was performed as described above. Active forms of MMP-2 and MMP-9 were examined.

STAT3 phosphorylation by OSM

Primary trophoblasts were seeded into six-well cell culture plates in DMEM/F12 medium supplemented with 10% FBS and cultured until they reached 70–80% confluency. Some cells were treated with OSM (20 ng/mL) for 5 min, 15 min, 30 min, 1 h, and 6 h under normoxic condition. Other trophoblast cells treated with or without OSM (20 ng/mL) were incubated for 6 h under normoxia or hypoxic condition. Western blot analysis was performed as described above, except that the following antibodies were used: mouse anti-human phosphorylated (phosphorylation of Tyr705) STAT3 (1:200) and mouse anti-human total STAT3 (1:50) (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Effect of OSM on MMP-2 and MMP-9 enzymatic activity under normoxia and hypoxia conditions

A gelatin zymography assay was performed as described previously.¹² Briefly, gel zymography with gelatin as a substrate was used to detect proteolytic activity of MMP-2 and MMP-9. Conditioned media from cell cultures were centrifuged. Supernatants were mixed with 5×SDS sample buffer (Fermentas, Burlington, Canada) and incubated at 37°C for 1 h. Samples were loaded onto a 10% gelatin Zymogram-PAG 10% pre-cast gel (Komabiotech, Seoul, Korea). To enable enzymes to renature, the gel was incubated twice in renaturation buffer (Komabiotech) for 20 min. It was then incubated with Zymogram development buffer (Komabiotech) for 20 min and fresh Zymogram development buffer at 37°C for 36 h. The gel was stained with Coomassie Blue R-250 (Komabiotech) for 30 min at room temperature and then destained twice with destaining solution for 15 min. To normalize protein amounts in the samples, we analyzed total protein by Coomassie staining.¹⁹ The presence of clear bands in gels at the appropriate molecular weights reflected the gelatinolytic activities of MMP-2 and MMP-9. Bands from gelatin zymography were quantified with Gel Doc XR_β with Image lab software (Bio-Rad).

Primary trophoblast cells pretreated with or without OSM (20 ng/L) for 24 h under normoxia and hypoxia conditions were used to check the enzymatic activities of MMP-2 and MMP-9 based on gelatin zymogram.

Effect of OSM on MMP-2 and MMP-9 enzymatic activities and STAT3 and pSTAT3 expression after STAT3 siRNA transfection

STAT3 siRNA transfection was performed as described in our previous study.¹³ Double-stranded siRNA oligonucleotide against STAT3 had a sequence of 5'-AATGTTCTCTATCAGCACAAT-3'.²⁰ Oligonucleotide was synthesized by Genolution Pharmaceuticals, Inc. (Seoul, Korea). Primary trophoblasts were seeded into six-well plates prior to transfection. For optimum transfection efficacy, cells were seeded to reach a final cell confluency of 30–50%. Cells were transfected with STAT3 siRNA (25 nM) (Genolution Pharmaceuticals, Inc., Seoul, Korea) for 24 h. After treatment with OSM (20 ng/mL) for 24 h, cells were dislodged from the surface of six-well culture plates for gelatin zymography.

Term trophoblasts were seeded into six-well cell culture plates in DMEM/F12 medium supplemented with 10% FBS and cultured until they reached 70–80% confluency. Primary trophoblasts were treated with OSM (20 ng/mL) for 24 h with or without pretreatment with STAT3 siRNA (25 nM) for 1 h in normoxia or hypoxia chamber. Control cells were incubated for 24 h under normoxia and hypoxia conditions without OSM or STAT3 siRNA treatment. Subsequent steps were the same as described above.

Invasion assay

Matrigel invasion assay was performed as described in our previous study.¹⁴ ECMatrix invasion by term primary trophoblasts with or without treatment of OSM (20 ng/mL) for 12 h and 24 h under normoxia and hypoxia conditions was determined. Cell counting was performed in triplicates. Data are expressed as mean±standard error of three experiments.

Statistical analysis

Data are expressed as mean±SEM. Non-parametric Mann-Whitney test was used to compare two groups. Statistical significance was considered at *p* value <0.05.

Institutional review board approval was obtained from the Catholic University Medical Center Institutional Review Board.

RESULTS

Effect of OSM on protein expression levels of MMP-2 and MMP-9 under normoxia and hypoxia conditions

By immunohistochemistry, we observed that the cultured cells expressed EVT markers, such as HLA-G. MMP-2 and MMP-9 proteins were constitutively expressed in term primary trophoblasts under normoxia and hypoxia conditions on Western blot (Fig. 2A). MMP-2 and MMP-9 protein expression induced by OSM (20 ng/mL) did not show significant changes until 24 h under normoxia condition (all *p*>0.050). However, OSM-induced MMP-2 protein expression was significantly in-

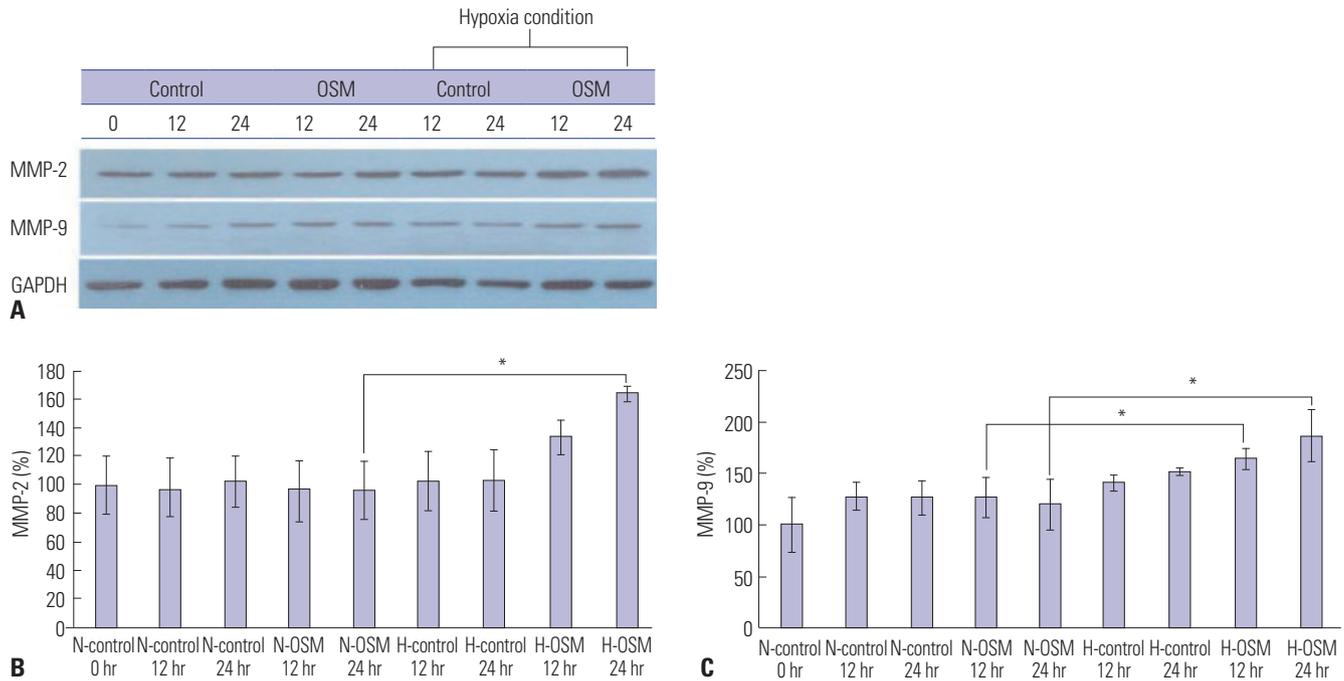


Fig. 2. Effect of OSM (20 ng/mL) treatment for 12 and 24 hours on MMP-2 and MMP-9 protein expressions in term trophoblasts (A) under normoxia and hypoxia. A quantitative measure of activities was determined by Western blot. Data shown are means \pm standard error of three independent experiments (B, C). The results are presented as the percent increase or decrease of protein expression with normalization to GAPDH. Significance versus untreated cells (control). * $p < 0.05$. OSM, oncostatin M; MMP, matrix metalloproteinase.

creased after 24 h ($p=0.016$), while MMP-9 protein expression induced by OSM was significantly increased after 12 h ($p=0.049$) and 24 h ($p=0.036$) under hypoxic condition (1–2% oxygen), compared with a sample incubated over the same timeline (Fig. 2B and C).

Effect of OSM on MMP-2 and MMP-9 enzymatic activities under normoxia and hypoxia conditions

Gelatin zymogram results showed the OSM (20 ng/mL) stimulated enzymatic activities of MMP-2 and MMP-9 in primary trophoblasts (Fig. 3A). Under hypoxia, the OSM-induced enzymatic activity of MMP-2 was significantly increased at 12 h ($p=0.040$) and 24 h ($p=0.016$), compared to that of normoxic control with OSM over the same timeline (Fig. 3B). After 12 h and 24 h of OSM treatment under hypoxia condition, the enzymatic activities of MMP-9 were significantly higher than those under normoxia condition with OSM at the same time ($p=0.003$ and $p=0.008$, respectively). The enzymatic activities of MMP-9 after 12 h and 24 h under the hypoxic condition were significantly increased, compared to those of the normoxia condition without OSM at the same time ($p=0.013$ and $p=0.010$, respectively). On the other hand, under normoxic condition, OSM did not show a stimulatory effect on the enzymatic activity of MMP-2 and MMP-9 with time, compared to normoxia control (all $p > 0.050$). OSM-induced enhancement in enzymatic activity of MMP-2 and MMP-9 under hypoxia condition was more pronounced, compared to that under normoxia condition (Fig. 3B and C).

STAT3 phosphorylation stimulated by OSM in primary trophoblasts

Total STAT3 protein expression did not change significantly after adding OSM (20 ng/mL) over time either under normoxia or hypoxia condition. Phosphorylated STAT3 protein expression in primary term trophoblasts was increased at 15 min after adding OSM (20 ng/mL) under normoxia condition ($p=0.018$). Under hypoxia condition, OSM-induced expression of phosphorylated STAT3 protein in term trophoblasts was also significantly increased after 15 min ($p=0.035$) (Fig. 4A and B).

Effects of OSM and STAT3 siRNA on MMP-2 and MMP-9 activities under normoxia and hypoxia conditions

After treatment with STAT3 siRNA, the OSM-induced activities of MMP-2 were significantly decreased, compared to OSM-stimulated expression of MMP-2 (both $p < 0.05$), regardless of oxygenation ($p=0.002$ at 12 h, $p=0.004$ at 24 h under normoxia and $p=0.005$ at 12 h, $p=0.006$ at 24 h under hypoxia, respectively). Also, OSM-induced enzymatic activities of MMP-9 were significantly decreased ($p < 0.001$ at 12 h, $p=0.002$ at 24 h under normoxia and $p=0.014$ at 12 h, $p=0.015$ at 24 h under hypoxia, respectively) (Fig. 4C and D). After treatment with STAT3 siRNA, OSM-induced total STAT3 protein expression was significantly decreased over time in both normoxia and hypoxia conditions (all $p < 0.001$). Also, phosphorylated STAT3 protein expression in primary term trophoblasts after STAT3 siRNA transfection was significantly depressed with OSM regardless of oxygen condition (all $p < 0.001$) (Fig. 4E and F).

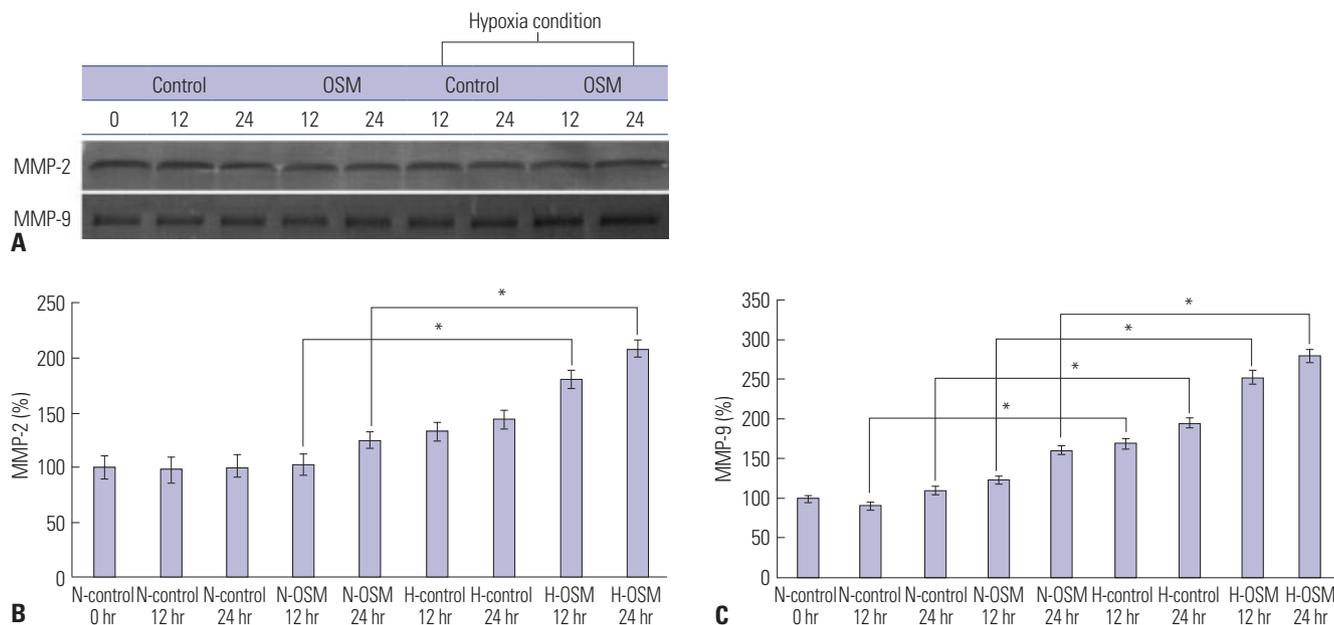


Fig. 3. Effect of OSM (20 ng/mL) treatment for 12 and 24 hours on MMP-2 and MMP-9 secretion in conditioned medium (A). A quantitative measure of activity was determined by gelatine zymography. Results are means±standard error of three experiments (B, C). * $p < 0.05$. OSM, oncostatin M; MMP, matrix metalloproteinase.

Effect of OSM on *in vitro* trophoblast invasiveness

Results of ECMatrix invasive assay revealed that the invasiveness of primary trophoblasts did not significantly increase with time under normoxia without OSM treatment. However, invasiveness was significantly increased after 24 h of treatment with OSM under hypoxia, compared to that of the normoxia condition with OSM ($p=0.012$) (Fig. 5). Under hypoxic condition, trophoblast invasiveness was significantly increased after 12 h without OSM treatment, compared with that of normoxia control at 12 h ($p=0.046$). The invasiveness of trophoblasts under hypoxic condition at 24 h after treatment with OSM was also significantly higher than that under the normoxia condition without OSM treatment at 24 h ($p=0.018$). Under hypoxia, trophoblast invasion according to OSM addition was significant increased at 24 h ($p=0.045$).

DISCUSSION

In early pregnancy, oxygen tension is known to be an important physiologic regulator of human hemochorial placentation.²¹ During the early first trimester (<10 weeks), trophoblast invasion occurs in a low oxygen environment because maternal blood flow is temporarily restricted by the endovascular plug. After 10 weeks of gestation when the endovascular plug is disintegrated and the spiral artery opens into the intervillous space, oxygen tension increases in decidua due to increased oxygenated maternal blood flow.^{7,21,22}

Using HTR8/SVneo cells, we previously demonstrated that OSM can induce the expression of MMP-2 and MMP-9, which are important for the invasion of EVT during early pregnan-

cy.¹² Our previous studies also suggested that STAT3 signaling pathways are related to OSM induced invasion of HTR8/SVneo cells.¹⁴ The present study demonstrated that, in primary term trophoblasts, OSM increased protein expression levels and enzymatic activities of MMP-2 and MMP-9 under low oxygen conditions. Unlike HTR8/SVneo cells where the effect of OSM on MMP-2 was more pronounced, the stimulatory effect of OSM on MMP-9 was more notable in primary trophoblasts.¹² Under normoxia condition, OSM showed a stimulatory effect on enzymatic activities of MMP-9. However, under normal oxygen condition, OSM did not show a stimulatory effect on enzymatic activities of MMP-2 in term primary trophoblasts. This might be due to the fact that MMP-9 is critical for human trophoblast invasion.²³ Regardless of oxygenation, OSM-induced expression of MMP-2 and MMP-9 in primary term trophoblasts was related to the STAT3 signaling pathway, a major signaling pathway of the IL-6 family observed in HTR8/SVneo cells.^{13,14}

Hypoxia itself showed a tendency to increase the invasiveness of term primary trophoblasts, although the increase was not statistically significant. OSM showed a stimulatory effect on the invasiveness of term trophoblasts at 24 h under hypoxia condition, compared to that under the normoxia condition. However, OSM did not significantly improve the invasiveness of primary trophoblasts in the hypoxic condition, compared to the hypoxia control without OSM. The increase of invasiveness was not obvious because term trophoblasts have reduced invasiveness, compared to trophoblasts in early pregnancy.¹⁷ OSM seems to be able to enhance primary trophoblast invasion under hypoxia similar to that in the early first trimester. Further studies are needed to confirm whether OSM affects trophoblast invasion in early trophoblasts and whether the effect of

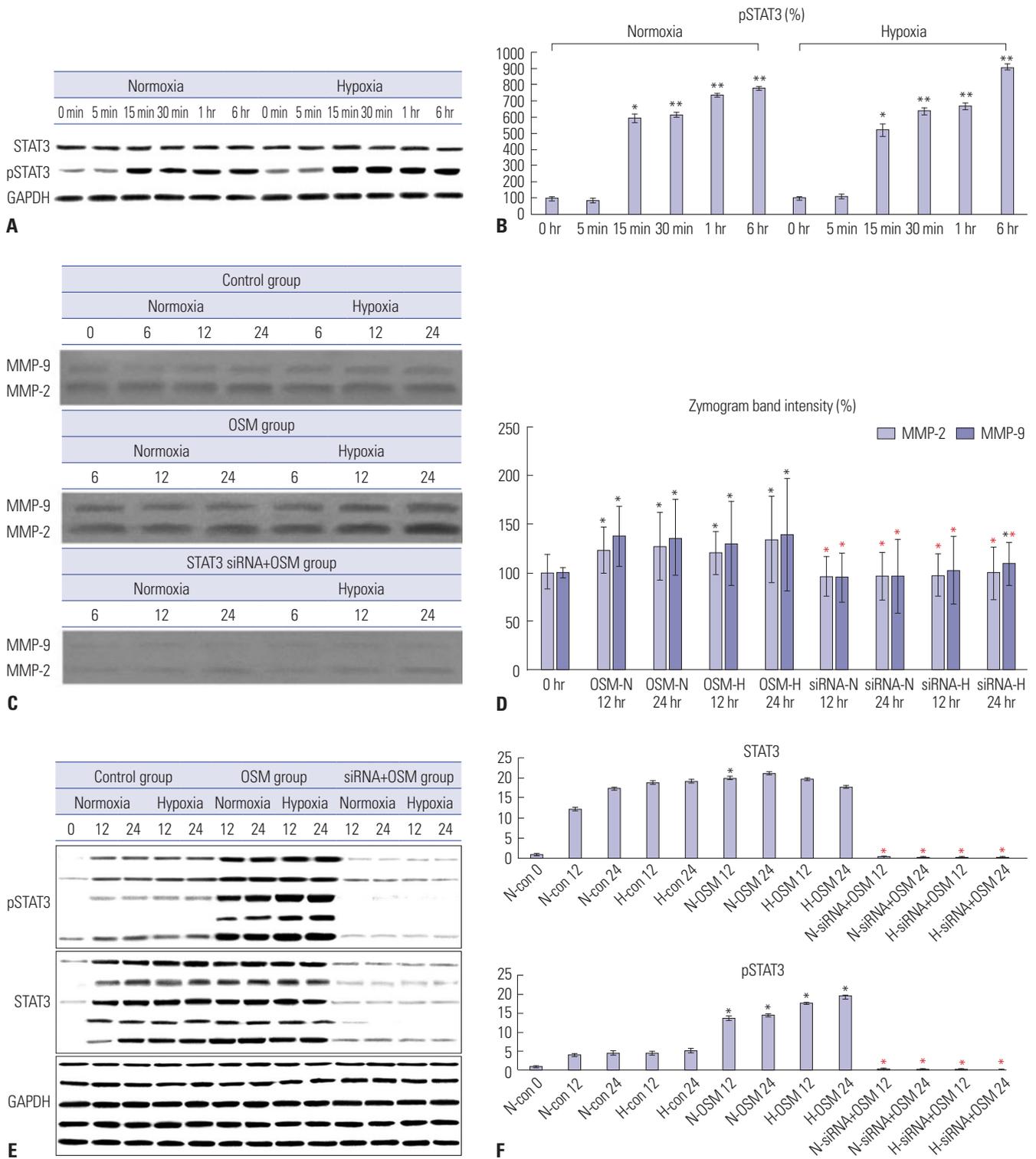


Fig. 4. Effects of OSM on protein expression of total and phosphorylated STAT3 (A). The expression was quantified and expressed as mean±SEM (B), **p*<0.05, ***p*<0.001. Effect of siRNA on MMP-2 and MMP-9 secretion in conditioned medium (C). A quantitative measure of activity was determined by gelatine zymography. The expression was quantified and expressed as mean±SEM (D), black **p*<0.05 (compared without OSM control at 0 hr), red **p*<0.05 (compared with OSM over the same time line). Effects of OSM on protein expression total and phosphorylated STAT3 after transfection of STAT3 SiRNA (E). STAT3 and pSTAT3 expression after STAT3 SiRNA transfection was quantified and expressed as mean±SEM (F), black **p*<0.05 (compared with normoxic or hypoxic control without OSM over the same time), red **p*<0.05 (compared with normoxic or hypoxic controls with OSM over the same time). The vertical axis represent the relative value when the value of normoxia control is set to 1.0. OSM, oncostatin M; MMP, matrix metalloproteinase; STAT, signal transducer and activator of transcription 3.

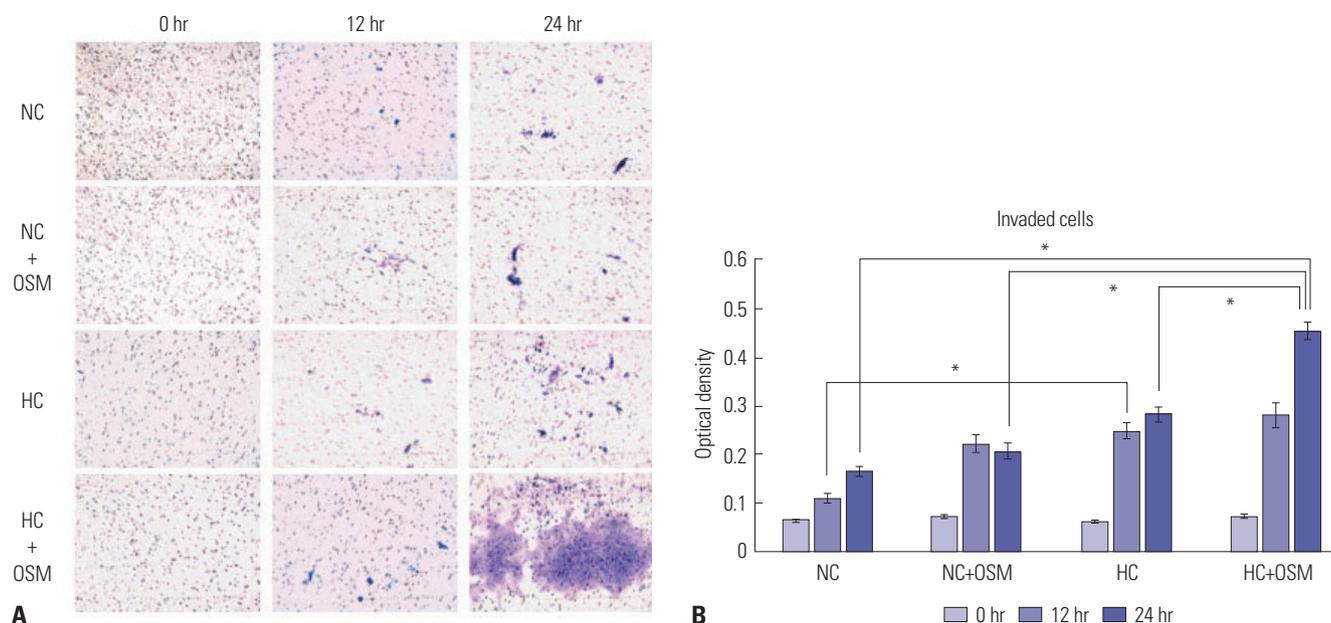


Fig. 5. EMatrix invasion by term primary trophoblasts ($\times 200$) after treatment with OSM (20 ng/mL) for 24 and 24 hours under normoxia (NC) and hypoxia (HC) (A). Results are presented as means \pm standard error of three experiments (B). * $p < 0.05$. OCM, oncostatin M.

OSM differs according to oxygenation condition.

Trophoblast invasion is affected by variable autocrine and paracrine factors, such as cytokine, hormone, growth factor, and oxygenation.²⁴ Hypoxia is known to be an important regulator of invasion of human trophoblast. However, results are conflicting.²¹ Many studies have shown that hypoxia itself tends to suppressed trophoblast invasion.^{6,22,25-27} Genbacev, et al.²⁵ have suggested that hypoxia can maintain non-invasive properties, enabling placental mass to increase in the early first trimester. After placental oxygen tension is increased by allowing maternal blood flow into the placental, the EVT differentiates into non-proliferative, invasive phenotype.²⁵ Increased oxygen results in down-regulation of hypoxia inducible factor-1 α and transforming growth factor to promote normal trophoblast invasion.²² On the other hand, several studies have shown that hypoxia may increase EVT invasiveness. Trophoblast-like cells, such as HTR-8/SVneo and JEG3 cells, exhibit increased invasiveness under low oxygen content.²⁸⁻³⁰ Our study showed that the expression levels and enzymatic activities of MMP-2 and MMP-9 and the invasiveness of term trophoblasts are enhanced under a low oxygen environment. OSM also showed a stimulatory effect on the expression and enzymatic activities of MMP-2 and MMP-9 in term trophoblasts under hypoxia.

Shallow trophoblast invasion is thought to be a pathogenic mechanism of preeclampsia and FGR.^{22,26} Persistent hypoxia after late first trimester might result in failure of trophoblast differentiation into invasive phenotype, shallow trophoblast invasion, and inadequate remodeling of the spiral artery.²² Impaired trophoblast invasion is also thought to be due to a reduced number of cells for invasion due to increased apoptosis or insufficient proliferation.²⁶ Proper proliferation during the early first trimester is essential for the establishment of ade-

quate placental mass and fetal growth.²⁵ Premature rise of placental oxygen, which may cause inadequate placental mass or spontaneous abortion, could reduce the proliferation of EVT in anchoring villi and cause a reduced number of EVTs available to invade the uterine wall and spiral.³¹ We previously demonstrated that the expression of OSM is increased in preeclamptic placenta.³² The present study demonstrated that the OSM-induced enzymatic activities of MMP-2 and MMP-9 are increased under a low oxygen environment. OSM might be able to increase the invasiveness of term EVTs under low oxygen content. This might lead to failure of achieving an adequate number of EVTs and shallow trophoblastic invasion. Another possibility is that increased expression of OSM in preeclampsia could be an adaptive phenomenon to compensate for impaired invasion of trophoblasts. Therefore, further studies are needed to investigate whether the expression of OSM is increased in the preeclamptic placenta and early trophoblasts under low oxygen concentration. Further study is also needed to determine whether trophoblast invasion and proliferation in preeclamptic placenta are affected by OSM.

Our study has some limitations. We cultured EVTs in a 1-2% hypoxic condition. Although this condition does not mimic the viable uterine condition, hypoxia elicited OSM-induced EVT invasion.

In conclusion, this study suggests that OSM enhances the protein expression levels and enzymatic activities of MMP-2 and MMP-9 under hypoxia. Its effects were found to be associated with the STAT3 pathway. Although there was no significant difference in the invasiveness of term primary trophoblasts by OSM under a normoxic condition, OSM-induced invasiveness was significantly increased under a low oxygen environment, compared to normoxia control, after 24 h of treatment

with OSM. This study suggests that the behaviors of term trophoblasts treated with OSM differ from those of HTR8/SVneo cells. However, the effects of OSM on early pregnancy trophoblasts and trophoblasts of adverse pregnancies, such as pre-eclampsia and FGR, need to be evaluated in future studies.

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