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Dynamic Alterations in Integrin α4 Expression by Hypoxia are Involved in Trophoblast Invasion During Early Implantation

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ABSTRACT

Implantation of the blastocyst into the maternal endometrium is mediated by a population of well-differentiated primary cells of the placenta known as trophoblasts, which grow in an invasive and destructive fashion similar to tumor cells. Interactions between the endometrium and trophoblasts are regulated by a coordinated interplay of extracellular matrix (ECM) proteins secreted by the invading extravillous trophoblasts. Integrins act as adhesion receptors and mediate both cell–ECM and cell–cell interactions. However, the correlation between integrin expression and trophoblast invasion under hypoxia is unclear. Here, we analyzed the expression of integrins in HTR-8/SVneo trophoblast cells exposed to hypoxic conditions in order to demonstrate an association between invasion activity and integrin expression in trophoblasts. Trophoblasts were examined by microarray analysis, RT-PCR, western blotting, and zymography after 1% hypoxic treatment, and cell invasion was estimated. The dynamic expression of integrins and human matrix metalloproteinases (MMPs) was observed under hypoxic conditions. The invasiveness of trophoblasts cultured under 1% hypoxic conditions was significantly greater than that of trophoblasts cultured under normoxic conditions through alterations in MMP-2 and -9 ($P < 0.05$). Notably, integrin α4 expression during early hypoxia was negatively regulated by hypoxia-inducible factor-1alpha (HIF-1alpha) expression in trophoblasts. The downregulation of integrin α4 expression by siRNA treatment controlled trophoblast invasion activity ($P < 0.05$). Taken together, we suggest that dynamic changes in integrins, including those in integrin α4 expression by hypoxia, play a regulatory role in trophoblast invasion. These findings expand our understanding of the potential roles of integrin α4 in implantation. J. Cell. Biochem. 113: 685–694, 2012.

KEY WORDS: INTEGRINS; HYPOXIA; TROPHOBLASTS; INVASION; IMPLANTATION

Implantation of the blastocyst into the maternal endometrium is mediated by differentiated extravillous trophoblasts derived from the trophectoderm in the outer layer of the blastocyst after fertilization [Red-Horse et al., 2004; Wang et al., 2007]. This culminates in controlled trophoblast invasion and successful implantation [Minas et al., 2005; Blomberg et al., 2008]. Extravillous trophoblast cells secrete large amounts of highly heterogeneous extracellular matrix (ECM) proteins along their invasion routes. These proteins are regulated by ECM receptors, including integrins, cell adhesion molecules, and gap junction molecules [Staun-Ram and Shalev, 2005; Fitzgerald et al., 2008]. Because the expression of various cell adhesion molecules is tightly regulated according to time, and because spatially different signaling patterns are necessary for normal placental development and embryogenesis, this process is complex, dynamic, and tightly controlled [Bowen and Hunt, 1999].

Integrins are receptors that mediate attachment between a cell and its surrounding tissues or ECM. As obligate heterodimers, integrins contain two distinct chains: alpha (α) subunits and beta (β) subunits. To date, 18 α and 8 β subunits have been characterized in

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Tae-Ki Yoon and Gi-Jin Kim contributed equally to this work.

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mammals [Humphries, 2000]. These subunits also play a role in cell signaling and thereby define cellular shape and mobility, and regulate cell fate [Hynes, 2002]. Many types of integrins exist and many cells have multiple types on their surface. Typically, receptors inform a cell of the molecules in its environment and the cell makes a response. The expression of several α integrin subunits, fibronectin, and vitronectin has been demonstrated during goat peri-implantation [Garcia et al., 2004]. In particular, integrin α5 is controlled by various factors, including insulin growth factor (IGF) and E-cadherin, during hypoxia. These factors function in the migration of extravillous trophoblast cells during early implantation via a cAMP-dependent mechanism [Kabir-Salmani et al., 2004; Coutifaris et al., 2005; Arimoto-Ishida et al., 2009]. Furthermore, α5,β3 integrin expression increases with hypoxia in the embryo and trophoblasts and induces successful implantation through an early embryo–maternal interaction. The IGF-activated α5,β3 signaling pathway triggers trophoblast migration and invasion [Kabir-Salmani et al., 2003; Cowden Dahl et al., 2005. Although the expression and function of various integrins in the embryo and maternal myometrium have been investigated in terms of their relationship with the mechanism of implantation, most studies have focused on the expression of limited integrin subunits such as α5,β3 and α5,β1; the roles of other integrin subunits are unclear.

Trophoblast invasion is tightly regulated by several factors, including the coordinated interplay of various growth factors, cytokines, hormones, and cell adhesion molecules, and is dependent on a hypoxic microenvironment. Hypoxia is a pathological condition indicating the lack of an adequate oxygen supply. Early placentation (<10–12 weeks of gestation) occurs in an environment characterized by relatively low concentrations of oxygen (17.9 mm Hg) as compared with the endometrium (39.6 mm Hg) [Kingdom and Kaufmann, 1999; Zygmunt et al., 2003]. Under hypoxic conditions, the trophoblast layer assumes a proliferative trophoblast phenotype and an invasive trophoblast phenotype, which may be important for achieving the depth sufficient for invasion [James et al., 2006]. Hypoxia-inducible factor-1alpha (HIF-1alpha) increases with hypoxia and facilitates the expression of a wide range of genes in response to hypoxia during implantation and the first trimester of pregnancy. Hypoxia appears to play a vital role in regulating trophoblast differentiation during the first trimester. Furthermore, constant hypoxic exposure alters cytotrophoblast differentiation and invasion during early gestation and may result in placental defects, including pre-eclampsia and intrauterine growth retardation in humans [Genbacev et al., 1996, 1997].

In the present study, we analyzed the expression of various integrins in HTR-8/SVneo trophoblast cells, which are dependent on exposure to hypoxia, and identified the association between integrin expression and trophoblast invasion activity. Furthermore, we examined the function of integrin α4 in HTR-8/SVneo trophoblast cells.

MATERIALS AND METHODS

CELL CULTURE

HTR-8/SVneo trophoblast cells, provided by Dr. C. H. Graham (Queen's University, Kingston, ON, Canada), were maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 5% foetal bovine serum (FBS; Invitrogen) and 100 U/ml penicillin and streptomycin (Gibco, Frederick, MD). To increase the efficiency of the hypoxic treatment, hypoxic medium was exposed to an atmosphere consisting of 99% N_2 and 1% O_2 for about 24 h. Six hours after seeding, the cells were given the hypoxic RPMI media and cultured in a hypoxia chamber (C-chamber; BioSpherix, Ltd., Laconia, NY) with a reduced atmospheric O_2 (1% O_2 and 99% N_2) for 12 and 24 h, respectively. At the end of the incubation period, the cells were collected and the pellets were maintained at −70°C until analysis.

SiRNA ASSAYS IN HTR-8/SVNEO TROPHOBLAST CELLS

Transfection experiments were performed with cytotrophoblast HTR-8/SVneo cells. The cells were cultured at 37°C and 5% CO_2 in RPMI-1640 (Invitrogen) supplemented with 5% FBS (Invitrogen) and 1% penicillin/streptomycin (Gibco) prior to transfection, which was conducted in Opti-Mem. All experiments were executed with cells at 50–60% confluence and seeded 24 h prior to transfection. Lipofectamine 2000 (Invitrogen) was used in all transfection experiments and was optimized independently for transfection efficiency with minimal cell toxicity. siRNAs (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were dissolved in 330 μl of diethylpyrocarbonate and stored at −20°C until the transfection experiment. The integrin α4 siRNA and HIF-1alpha siRNA consisted of a pool of three target-specific siRNAs 20–25 nt in length that were designed to knock down gene expression. The concentrations of integrin α4 siRNA (two concentrations) and HIF-1alpha siRNA were 50 pM and 100 pM, and 20 nM, respectively. All samples were incubated for 48 h.

MICROARRAY ANALYSIS

A high-density human whole-genome oligonucleotide gene chip (Illumina Human HT-12 v3; Illumina, Inc., San Diego, CA) set was used. This set contained 48,803 probes targeting human genes. Total RNA was extracted from HTR-8/SVneo trophoblast cells with and without hypoxia treatment using TRIzol reagent following the manufacturer's protocol (Life Technology, Rockville, MD). In our microarray analyses, 0.8 μg of total RNA was used to make biotin-labeled cRNA with an Ambion Illumina cRNA amplification and labeling kit according to the manufacturer's instructions (Ambion, Austin, TX). The cRNA quality was verified using Experion (Bio-Rad, Hercules, CA) prior to hybridization. The biotin-labeled cRNA was labeled with fluorescent dye at the Rockefeller University Gene Array Facility and hybridized onto an expression array bead chip. The arrays were then scanned using an Illumina Bead Station laser scanning imaging system; an average of 30 beads/gene transcript was used to generate the expression data. Data analysis was performed using GenPlex software 3.0 (Istech Inc., Seoul, Republic of Korea) and quantile normalization was used to normalize the data. For primary data filtering, spots with a P-call (detection *P*-value < 0.1) were selected, and the remaining filtered data were used for further analysis. Hierarchical clustering of log ratios was performed using Cluster and TreeView 2.3; Euclidean correlation, median centring, and average linkage were applied during all clustering applications. An analysis of our time-course data was

performed with Microsoft Excel, as described previously [Nam et al., 2009].

SEMIQUANTITATIVE RT-PCR (QRT-PCR)

Total RNA was isolated from HTR-8/SVneo cells exposed to hypoxic conditions and HTR-8/SVneo cells after treatment with integrin α4 siRNA for 48 h. Total RNA (1 μg) was used for reverse transcription using Superscript III reverse transcriptase (Invitrogen). cDNA was amplified using hTaq polymerase (Solgent, Daejeon, Republic of Korea). The primers used were designed based on the sequences in humans (Table I). The following conditions were employed for amplification: A 15-min presoak at 94°C followed by 35 cycles of denaturation for 20 s at 94°C, annealing for 40 s at 55–62°C, and extension for 1 min at 72°C, with an additional 5-min incubation at 72°C after completion of the programme. The amplified cDNA fragments were electrophoresed on a 1% agarose gel, stained with 0.5 μg/ml ethidium bromide (Promega, Madison, WI), and visualized with a video image analyser (Bio-Rad). The genes of interest and 28S rRNA as a control were quantified by densitometry using Quantity One software (Bio-Rad). The data are presented as the ratio of the optical density of the gene of interest to that of 28S rRNA.

WESTERN BLOT ANALYSIS

Cell lysates were collected from HTR-8/SVneo cells exposed to hypoxic conditions and HTR-8/SVneo cells after treatment with integrin α4 and HIF-1alpha siRNA for 48 h. Cells were washed with cold Dulbecco's phosphate-buffered saline (PBS) and lysed in 300 μl of cold cell lysis buffer (Fermentas, Burlington, ON, Canada). The cell pellets were homogenized and sonicated in protein extraction solution (Intron, Kyunggi, Republic of Korea) and centrifuged at 12,000 rpm for 15 min at 4°C. The total protein concentrations of the supernatants were determined with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Total protein extracts (40 μg) were heated at 95°C for 5 min, resolved by 12% SDS-PAGE and electrotransferred to polyvinylidene fluoride membranes at 100 V for 80 min. The blots were blocked for 1 h in PBS (10 mM Tris-HCl, pH 7.5, and 150 mM NaCl) containing 5% nonfat dry milk (Becton Dickinson, Franklin Lakes, NJ) and 0.05% Tween 20 at room temperature. The blots were then washed three times with PBS-T and incubated at 4°C overnight with primary antibodies specific for human HIF-1alpha (1:1,000 dilution, provided Dr. JW Park, Seoul National University College of Medicine, Seoul, Korea), integrin α4 (1:1,000 dilution; Prosci, Poway, CA), integrin α5 (1:2,000 dilution; R&D Systems, Minneapolis, MN), integrin β1 (1:1,000 dilution; R&D Systems), integrin β7 (1:1,000 dilution; R&D Systems), or β-actin (1:1,000 dilution; Santa Cruz Biotechnology Inc.), followed by horseradish peroxidase-conjugated rabbit (1:5,000) or mouse (1:10,000) secondary antibodies (Bio-Rad). Peroxidase activity was visualized with an ECL Advance Western blotting detection kit (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK).

ZYMOGRAPHY

The gelatinolytic capacities of matrix metalloproteinase (MMP)-2 and -9 were determined by gelatin zymography. Each tissue protein sample (5 μg) was mixed with SDS sample buffer and loaded onto a 12% polyacrylamide gel containing 0.1% gelatin under nonreducing conditions. After electrophoresis, the gels were renatured with renaturation buffer (Bio-Rad) for 30 min at room temperature and incubated with development buffer (Bio-Rad) for 16 h at 37°C. The gels were subsequently stained with 0.5% Coomassie Brilliant Blue G-250 dye solution for 1 h at room temperature then destained with a buffer consisting of 30% methanol, 10% acetic acid, and 60% deionized water for 3 h to visualize the zymogen bands. The zones of gelatinolytic activity were revealed by negative staining and the gels were scanned. The molecular size of the bands displaying enzymatic activity was estimated using prestained standard protein molecular weight markers (GenDepot Inc., Barker, TX).

TABLE I. The Sequences for Specific PCR Primer Pairs and the Different Parameters for PCR Amplification

Gene	Sequences	Tm (°C)	Size (bp)
Integrin-α3	F: 5′-AAGCCAAGTCTGAGACTGTG-3′ R: 5′-CAGGGAGGACGTCAGTAGTA-3′	58	650
Integrin-α4	F: 5′-CAGACGTGCGAACAGCAGCTCCAG-3′ R: 5′-GCCAGTCCAGTAAGATGATCCTGG-3′	55	450
Integrin-α5	F: 5′-CATTTCCGAGTCTGGGCCAA-3′ R: 5′-TGGAGGCTTGAGCTGAGCTT-3′	65	320
Integrin-α6	F: 5′-TCCCTGAACCTAACGGAGTCT-3′ R: 5′-ATGTCCAAGTAGTTCAGTTTG-3′	52	250
Integrin-β1	F: 5′-GTCTTGGAACAGATCTGATGAAT-3′G R: 5′-TTTCTGGACAAGGTGAGCAATAG - 3′	62	500
Integrin-β7	F: 5′-AGCAGCAACAACTCAACTGG-3′ R: 5′-TTACAGACCCACCCTTCCTCT-3′	56	189
HIF-1a	F: 5′-TGGACTCTGATCATCTGACC-3′ R: 5′-CTCAAGTTGCTGGTCATCAG-3′	50	434
MMP-2	F: 5′-CGGCCGCAGTGACGGAAA-3′ R: 5′-CATCCTGGGACAGACGGAAG-3′	54	211
MMP-9	F: 5′-GACGCAGACATCGTCATCCAGTT-3′T R: 5′-GCCGCGCCATCTGCGTTT-3′	154	200
TIMP-1	F: 5′-TACTTCCACCGGTCCCACAACC-3′ R: 5′-GGCTATCTGGGACCGCAGGGACTGCCA-3′	68	339
TIMP-2	F: 5′-CTCTGGAAACGACATTTATGGC-3′ R: 5′-AGATGTAGCACGGGATCATGGG-3′	57	332
28s rRNA	F: 5′-TTGAAAATCCGGGGGAGAG-3′ R: 5′-ACATTGTTCCAACATGCCAG-3′	52	100

INVASION ASSAY

A Matrigel invasion assay was performed in 24-well filtered inserts with membranes (pore size 8 μm; Costar, Cambridge, MA). HTR-8/SVneo trophoblast cells (1×10^5) and HTR-8/SVneo trophoblast cells (1×10^5) transfected with integrin α4 siRNA were plated in Transwell inserts pre-coated with Matrigel (thawed at 4°C overnight and diluted to 1 mg/ml; BD Biosciences and Beit-Haemek, Evron, Israel) and incubated with serum-free RPMI-1640 medium. The lower well was used with the same medium containing 10% FBS. After a 24-h incubation, the cells in the upper wells were removed completely with a cotton swab, and the cells that had attached to the bottom side of the filter were fixed with methanol and stained with hematoxylin and eosin. Cell invasion ability was determined by counting the number of stained cells attached to the other side of the filter in 10 randomly selected nonoverlapping fields at a magnification of ×200. Invasion of the cells under different conditions was normalized to the control and expressed as the mean invasion (% invasion ± SEM).

STATISTICS

The data, which are presented as the mean ± SEM, were analyzed using an analysis of variance (ANOVA) followed by Duncan's multiple comparison test with $P < 0.05$ for statistical significance. All experiments were performed in duplicate and were repeated at least three times. For the microarray analysis, data were normalized using Global Shift normalization. Spots <50 μm were excluded from the analysis, unless otherwise specified. The Cluster and TreeView programmes were used for data visualization [Nam et al., 2005].

RESULTS

To determine whether genes regulated in HTR-8/SVneo trophoblast cells by hypoxia were associated with trophoblast invasion, we prepared expression profiles using an Illumine human HT-12 v3 gene chip containing 48,800 probes representing approximately 37,801 unique human genes. In an unsupervised hierarchical cluster analysis, we confirmed the expression profiles of HTR-8/SVneo trophoblast cells exposed to hypoxia with 156,979 genes that passed the basic filtering criteria described in the Materials and Methods section. Finally, we collected the expression profiles of HTR-8/SVneo trophoblast cells exposed to hypoxia with 835 genes that showed twofold changes for all array selection criteria (Fig. 1A and Supplementary Data 1). In the gene profiles, 249 genes were related to KEGG pathway signaling, focal adhesion, ECM-receptor interactions, and other functions (Table II). This resulted in four groups after hypoxia treatment: Group I (185 genes) contained gene profiles that increased within 4 h; group II (322 genes) contained gene profiles that increased after 4 h; group III (133 genes) contained gene profiles that decreased within 4 h; and group IV (191 genes) contained gene profiles that decreased after 4 h (Fig. 1B and Supplementary Data 1). Most integrins, except integrin α4, were found in group II, which increased 4 h after HTR-8/SVneo trophoblast cells were exposed to hypoxic conditions. Furthermore, integrin β1, MMP-9, and TIMP-1 expression was consistently upregulated after hypoxia treatment (Fig. 1C). Also, HIF-1alpha mRNA expression in HTR-8/SVneo trophoblast cells exposed to hypoxic conditions increased at 4 h; however, it rapidly decreased after 8 h under hypoxic conditions. This finding suggests that the genes expressed in HTR-8/SVneo trophoblast cells exposed to hypoxia changed dynamically and that their alteration was associated with various cellular events, including adhesion and trophoblast invasion.

To study the integrin family, which is the family most associated with trophoblast cell invasion, in detail, we validated our gene expression data using semiquantitative RT-PCR and western blot analysis. By semiquantitative RT-PCR, integrins α3 and β1 were found to be consistently expressed regardless of the hypoxic condition; the expression of integrins α5 and α6 increased gradually in a hypoxia-dependent fashion. Integrin α4 expression was very weak at the earliest time point, but its expression increased at the latest time point (Fig. 2). In agreement with our microarray and semiquantitative RT-PCR results, HIF-1alpha, integrin α5, and integrin β1 expression in HTR-8/SVneo trophoblast cells increased early on, but gradually decreased after hypoxia treatment, whereas integrin α4 and integrin β7 expression increased at the latest time point after exposure to hypoxia (Fig. 3A). Although many of the integrins were dynamically expressed in HTR-8/SVneo trophoblast cells exposed to hypoxia, integrin α4 expression was weak until 8 h after hypoxia treatment (Fig. 3B). These results suggest that HIF-1alpha expression under hypoxic conditions is associated with the dynamic expression patterns of integrins in HTR-8/SVneo trophoblast cells.

Next, we conducted a Matrigel invasion assay using HTR-8/SVneo trophoblast cells exposed to hypoxic conditions. No significant differences in trophoblast invasion occurred during normoxia or hypoxia until 2 h; however, trophoblast invasion increased significantly from 4 h after hypoxia treatment as compared with normoxic treatment (Fig. 4A,B; $P < 0.05$). To determine whether hypoxic conditions were correlated with trophoblastic invasion, we analyzed the expression of MMP-2, MMP-9, TIMP-1, and TIMP-2, which are involved in trophoblast invasion and were altered in our microarray data for HTR-8/SVneo trophoblast cells exposed to hypoxia. MMP-2 and TIMP-2 expression was constant, but MMP-9 and TIMP-1 expression increased in a hypoxia-dependent manner (data not shown). Next, we analyzed the activity of MMP-2 and -9 secreted from HTR-8/SVneo trophoblast cells exposed to hypoxia using zymography. During normoxia, the activities of MMP-2 and -9 in HTR-8/SVneo trophoblast cells were consistently maintained; however, the activity of MMP-2 and -9 in HTR-8/SVneo trophoblast cells after hypoxia increased. Specifically, MMP-9 activity was strongly increased (Fig. 4C). These findings are similar to those from our microarray analysis. These results indicate that HTR-8/SVneo trophoblast cell invasion is affected by hypoxia and that trophoblast cell invasion increased significantly at 4 h after hypoxia treatment. Furthermore, altered MMP activity and expression in HTR-8/SVneo trophoblast cells may be involved in trophoblast cell invasion.

To explore whether altered integrin α4 expression is associated with the genetic regulation of trophoblast invasion, we used a trophoblast invasion assay based on siRNA-mediated integrin α4

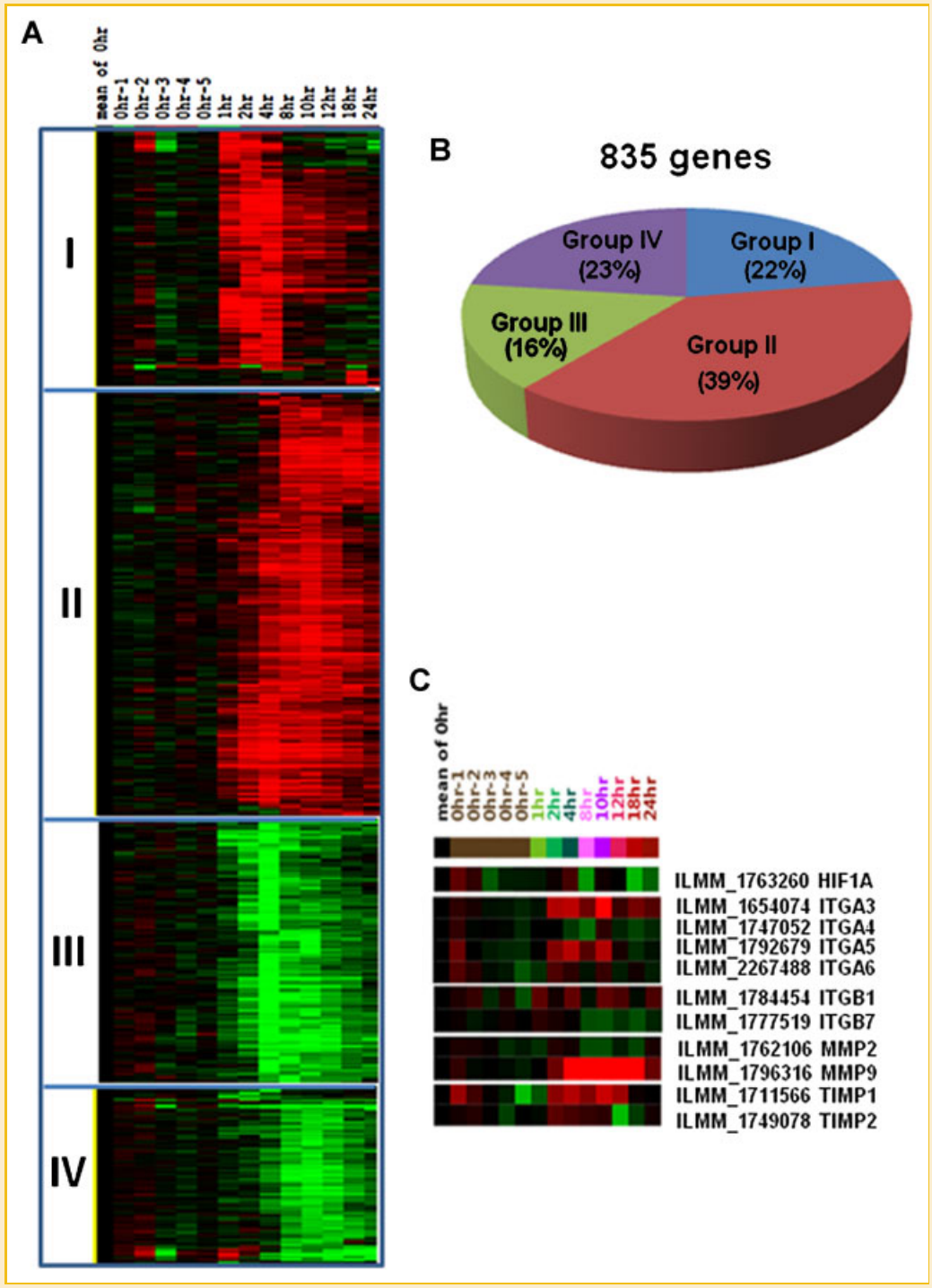

Fig. 1. Hierarchical clustering identifies hypoxia-induced trophoblast cells (HTR-8/SVneo) versus controls. A: In total, 835 genes showed twofold changes for all array selection criteria. This resulted in four groups approximately 4 h after hypoxia. B: Group I (185 genes) contained gene profiles that increased within 4 h, group II (322 genes) contained gene profiles that increased after 4 h, group III (133 genes) contained gene profiles that decreased within 4 h, and group IV (191 genes) contained gene profiles that decreased after 4 h. C: Expression of invasion-related genes in trophoblast cells exposed to hypoxic conditions. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

knockdown. No differences in cell viability were observed between the siRNA-mediated integrin α4 knockdown and HTR-8/SVneo trophoblasts (Fig. 5A). Integrin α4 expression in HTR-8/SVneo trophoblasts decreased after 100 pM siRNA transfection; otherwise, no differences were seen in the expression of integrins β1 and β7, which bind integrin α4 (Fig. 5B). Interestingly, integrin α4 expression increased significantly in the trophoblasts after HIF-1alpha siRNA treatment. In contrast, integrin β1 expression

TABLE II. KEGG Pathway Analysis

KEGG pathway	Gene Counts
Cytokine-cytokine receptor interaction	30
MAPK signaling pathway	28
Pathways in cancer	26
Focal adhesion	22
Glycolysis/Gluconeogenesis	22
Jak-STAT signaling pathway	16
Hematopoietic cell lineage	14
ECM-receptor interaction	13
Purine metabolism	13
Apoptosis	13
p53 signaling pathway	11
Cell cycle	11
Toll-like receptor signaling pathway	10
Axon guidance	10
Neuroactive ligand-receptor interaction	10

P-value = 0.0001.

decreased slightly (Fig. 5C). These data suggest that integrin α4 is negatively regulated by HIF-1alpha.

Furthermore, invasion in trophoblasts exposed to 50 and 100 pM integrin α4 siRNA increased significantly under normoxic conditions (Fig. 5C; $P < 0.01$). Interestingly, no differences in trophoblast invasion were observed for the integrin α4 siRNA-treated cells under hypoxic conditions. These findings suggest that integrin α4 downregulation in trophoblasts induces an increase in trophoblast invasion activity under normoxic conditions, but not under hypoxic conditions. Moreover, it is possible that integrin α4 is negatively regulated by HIF-1alpha expression in trophoblasts.

DISCUSSION

During early pregnancy, trophoblast cells within the placenta must complete complex biological processes for implantation. Trophoblasts derived from the trophectoderm, the outermost epithelial cell layer of the blastocyst, trigger attachment and implantation into the maternal endometrium through interactions between several factors (e.g., leukemia inhibitory factor and adhesion molecules) and the uterine microenvironment. Differentiation of the trophoblast from a cytotrophoblast to a syncytiotrophoblast is necessary once the trophoblast attaches to the endometrium; this process occurs within 2–3 days [Staun-Ram and Shalev, 2005]. Successful differentiation into a syncytiotrophoblast promotes adhesion of the blastocyst to the luminal epithelium of the endometrium, and integrins secreted from the trophectoderm help the trophoblast adhere and invade. Finally, the extravillous trophoblast invades the maternal myometrium, remodels the spiral arteries in the myometrium, and increases blood flow toward the intervillous space during placental development [Red-Horse et al., 2004]. Therefore, oxygen tension in the myometrium when the blastocyst attaches is low (~2–3% O_2) until the spiral arteries are transformed [Kingdom and Kaufmann, 1999; Zygmunt et al., 2003]. These microenvironmental conditions trigger vasculogenesis, trophoblast invasion and the secretion of several growth factors and cytokines from trophoblast cells. Many studies have examined gene expression during implantation in humans to clarify the mechanisms of implantation [Franchi et al., 2008; Gaide Chevronnay et al., 2009. In addition, the correlations between hypoxia and trophoblast invasion in an in vitro model or in vivo samples using high-throughput analysis led to successful implantation and the maintenance of pregnancy [Cammas et al., 2005; Roh et al., 2005]. However, it is difficult to evaluate the functions of known genes related to implantation and the mechanism of implantation via trophoblast invasion.

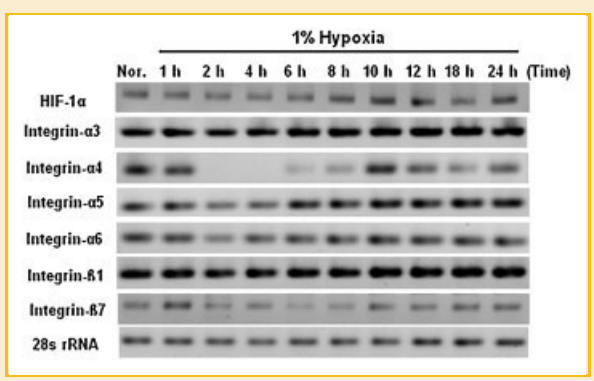

Fig. 2. RT-PCR analysis of the expression of integrins in HTR-8/SVneo trophoblast cells showing the dependency on exposure time under hypoxic conditions. Integrins α3 and β1 were constantly expressed regardless of the hypoxic condition; the expression of integrins α5, α6, and β7 increased gradually dependent on hypoxia. In contrast, integrin α4 expression was very weak at the earliest time point, but its expression increased at the latest time point. 28s rRNA was used as an internal control.

HIFs regulate an adaptive transcriptional response to hypoxia, which is a representative environmental factor that occurs during implantation and placental development [Wang and Semenza, 1993; Genbacev et al., 1996; Wenger and Gassmann, 1997; Ietta et al., 2006]. Alterations in HIF expression, and especially HIF-1alpha, seem to be involved in determining cell fate and activating MMP-1, which is associated with ECM degradation and cancer cell invasion [Lash et al., 2002; Sun et al., 2010]. When extravillous trophoblasts invade the myometrium, degradation of the endometrial ECM is regulated by various proteinases (MMPs), plasminogen activators, and tissue inhibitors [Das et al., 2002; Tapia et al., 2008]. Previous reports have suggested that HIF-1alpha plays an important role during early pregnancy. In particular, low O_2 tension is a critical determinant of trophoblast fate and function, and of the expression of several genes during placentation [Wyatt et al., 2005; Huppertz et al., 2009]. Furthermore, we reported that increased immortalization-upregulated protein 2 (IMUP-2), a novel protein found in trophoblasts under hypoxic conditions, induces preeclampsia and apoptosis in trophoblasts [Jeon et al., 2010]. Therefore, hypoxic conditions during implantation and early pregnancy are one of the most important environmental factors dynamically regulating gene expression. Alterations in the expression of various genes in trophoblast cells exposed to hypoxia were examined by cDNA microarray analysis (Supplementary Data 1). HIF-1alpha is hydroxylated at conserved proline residues by HIF prolyl-hydroxylases and is rapidly degraded by the proteasome under normoxic conditions. In contrast, it is stable under hypoxic conditions because HIF prolyl-hydroxylases are inhibited [Genbacev et al., 1996; Kvietikova et al., 1997]. It has been reported that

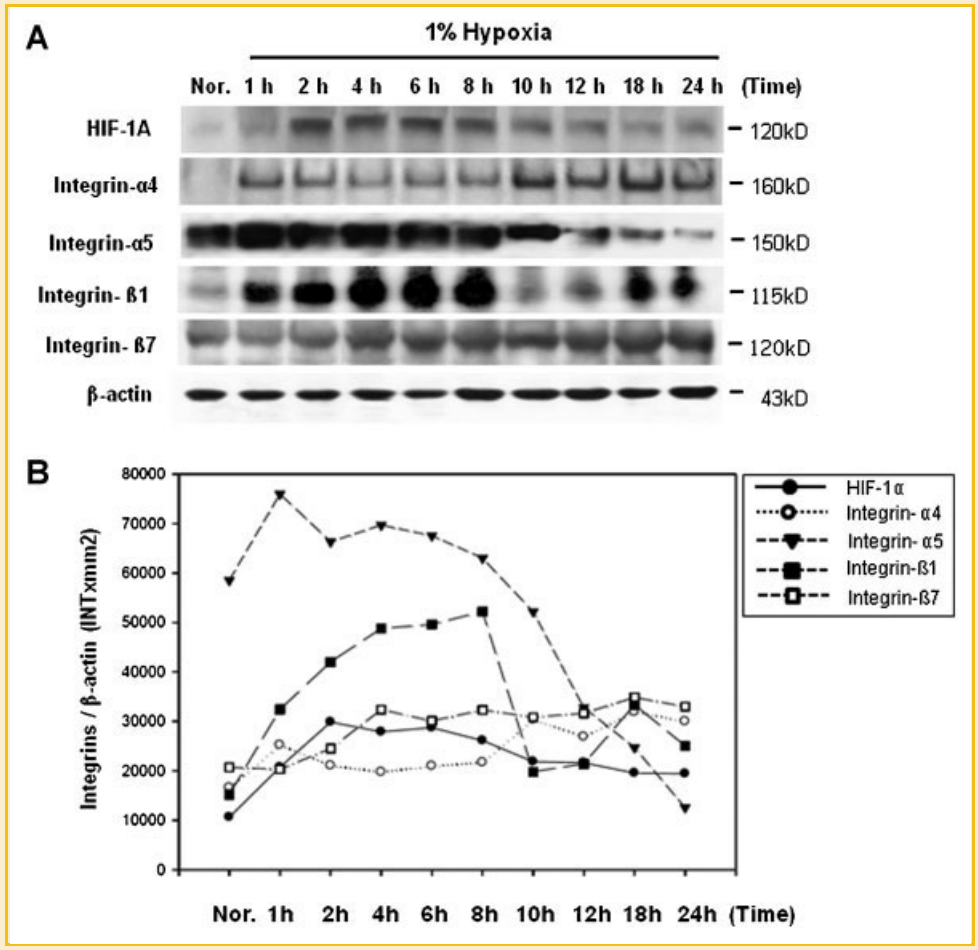

Fig. 3. Western blot analysis of the expression of integrins in HTR-8/SVneo trophoblast cells showing the dependency on exposure time under hypoxic conditions. A: HIF-1alpha, integrin α5, and integrin β1 expression in HTR-8/SVneo trophoblast cells increased early on, but gradually decreased after hypoxia treatment. In contrast, integrin α4 and integrin β7 expression increased at the latest time point after exposure to hypoxia. B: Comparative expression patterns of integrins in HTR-8/SVneo trophoblast cells exposed to hypoxic conditions. The integrins were dynamically expressed in HTR-8/SVneo trophoblast cells based on hypoxia treatment. β-actin was used as an internal control.

ERK1/2 phosphorylation and Ras/MAP kinase-mediated signaling pathways are associated with HIF-1alpha expression [Sheta et al., 2001; Qian et al., 2004; Sun et al., 2010]. Generally, the half-life of HIF-1alpha is very short, resulting in degradation within minutes after restoring normoxic conditions. This observation is consistent with our microarray and western blot data (Figs. 1C and 2B).

In the present study, we focused on integrin-related gene expression in trophoblast cells exposed to hypoxia because their dynamic expression depends on the time course after hypoxic treatment. Here, we demonstrated a correlation between integrin α4 and trophoblast invasion. This is the first report in humans on implantation from the trophoblast perspective and the association between integrin α4 expression and trophoblast invasion based on oxygen conditions. The relevance of these genes is emphasized because they were expressed during trophoblast invasion and peri-implantation. In particular, integrins, which are a family of cell adhesion molecules, are heterodimers consisting of α and β subunits, and the ligands are determined by the subunit combinations [Hynes, 2002]. The regulation of integrin α5 by E-cadherin loss through focal adhesion kinase (FAK) phosphorylation under hypoxic conditions and by cell–cell adhesion via cAMP-dependent mechanisms is important for the migration of extravillous trophoblasts during early implantation and affects normal placental development. The upregulation of integrin α5 can induce critical gynecological disorders such as preeclampsia [Coutifaris et al., 2005; Arimoto-Ishida et al., 2009].

In our microarray and western blot analyses, integrin α5 upregulation was observed from the earliest time point after hypoxia treatment (Figs. 1C and 3A). Notably, the level of integrin α5 expression was higher than that of other integrin subunits. Increased integrin α5, which binds to integrin β3, is associated with embryo–endometrium interactions during the early implantation stage through the induced migration of extravillous trophoblasts [Kabir-Salmani et al., 2004]. In addition, integrin β1, which is a major integrin β subunit, binds several integrin α subunits, which also increased at the earliest time point after hypoxia treatment [Nardo et al., 2003; Iwaki et al., 2004].

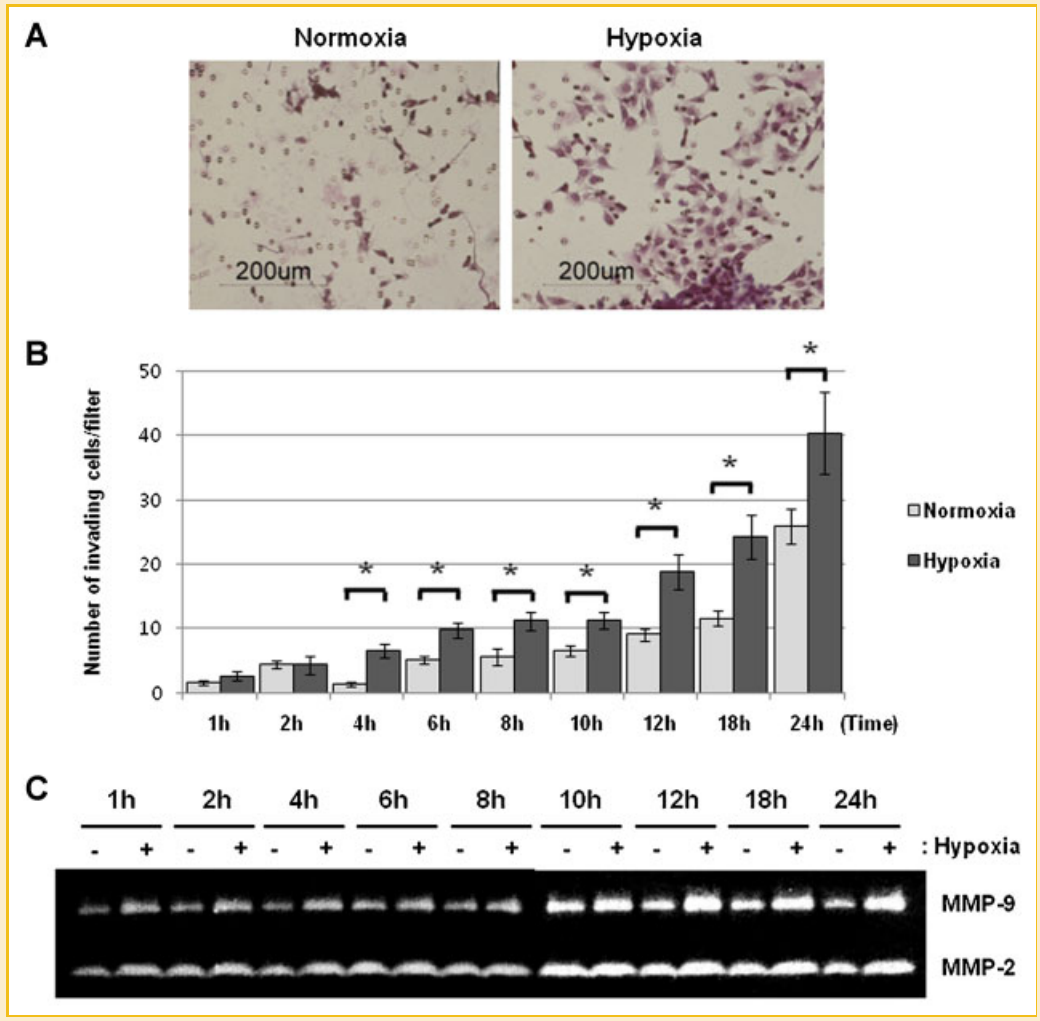

Fig. 4. Invasion assay in HTR-8/SVneo trophoblast cells exposed to 1% hypoxia. A: Comparison of HTR-8/SVneo trophoblast cell invasion between normoxic and hypoxic conditions at 24 h. Arrows indicate invading trophoblast cells. Scale bars: 200 μm. B: Number of invading cells in HTR-8/SVneo trophoblast cells between normoxic and hypoxic conditions. C: MMP activities in HTR-8/SVneo trophoblast cells exposed to 1% hypoxia as revealed by zymography. MMP-9 activity increased strongly, whereas MMP-2 activity was maintained. The results, presented as means ± SEM, were produced by an ANOVA followed by Duncan's multiple comparison test with $P < 0.05$ as an indicator of significance. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

Integrin α4 expression was weak at the early stage of hypoxia, although its expression increased during hypoxia. Integrin α4 expression is regulated by the spatial and temporal expression of GCMa, a glial cell missing gene transcription factor, and the expression of EMILIN1, a connective tissue glycoprotein associated with elastic fibers in the decidual stroma. This regulation effectively promotes cell adhesion and the migration of trophoblasts to blood vessels via the α4,β1 integrin trophoblast receptor [Spessotto et al., 2006; Schubert et al., 2008]. Moreover, integrin α4 expression decreased when HIF-1alpha expression increased. In contrast, at other times it increased when HIF-1alpha expression was down-regulated. As noted above, the expression of HIF-1alpha could alter integrin α4 expression, including integrins α5, β1, and β7 (Fig. 3A). In particular, integrin α4 expression might be negatively regulated by HIF-1alpha expression during the early stages of hypoxia (Fig. 5C). In addition, the invasion activities of trophoblasts downregulated by integrin α4 expression via integrin α4 siRNA treatment during normoxia increased significantly as compared with the control (Fig. 5D; $P < 0.001$). At other times, no differences were observed under hypoxic conditions regardless of integrin α4 siRNA treatment. These findings indicate that integrin α4 down-regulation could promote trophoblast invasion.

In conclusion, integrins in the trophoblast were dynamically expressed during hypoxia, and the expression of integrin α4 during early hypoxia was negatively regulated by HIF-1alpha expression in trophoblasts. In addition, the invasiveness of trophoblasts cultured under 1% hypoxia increased significantly as compared to those cultured under normoxic conditions. Taken together, these results suggest that dynamic alterations in integrins, including integrin α4 expression during hypoxia, help regulate trophoblast invasion and contribute to our understanding of the potential roles of integrin α4 in implantation.

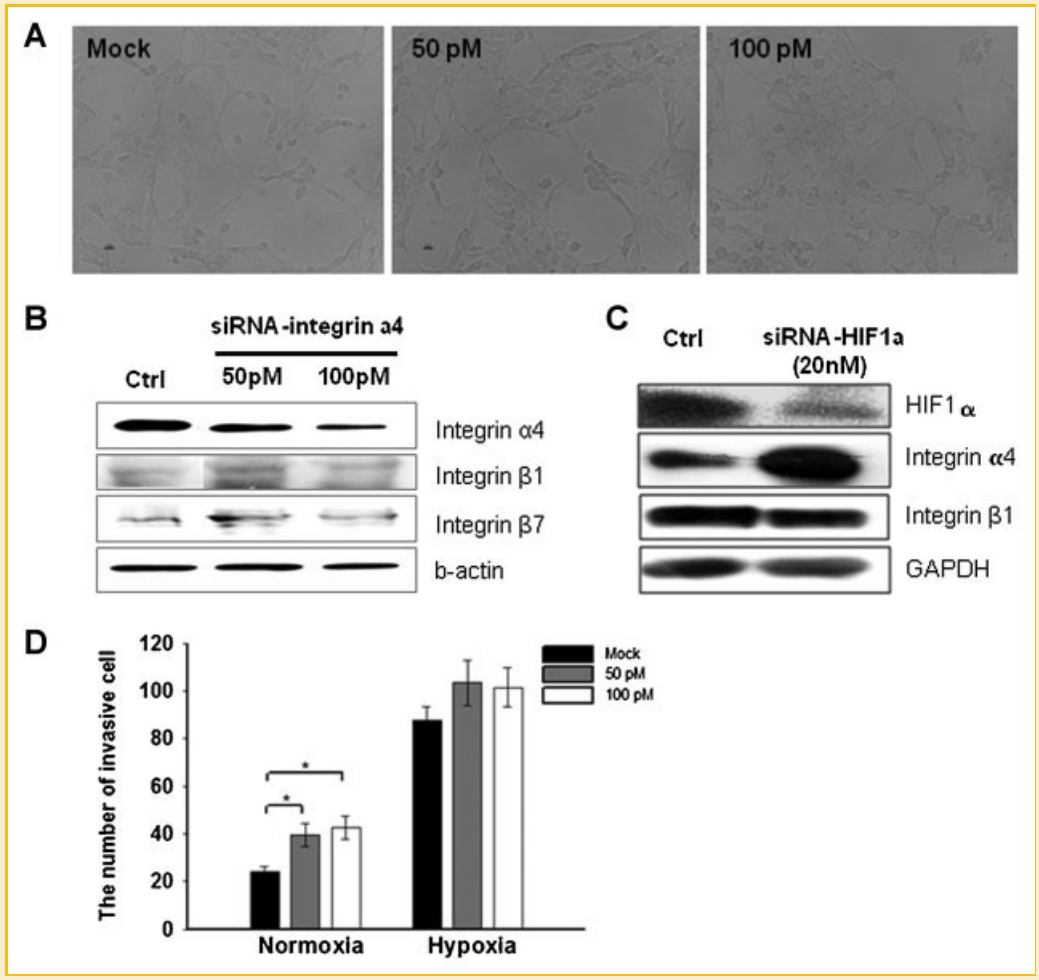

Fig. 5. Correlation between the downregulation of integrin α4 and invasion activity in HTR-8/SVneo trophoblast cells under hypoxia. A: Morphology of HTR-8/SVneo trophoblast cells after integrin α4 siRNA treatment for 24 h. B: Western blot analysis of integrin expression after integrin α4 siRNA treatment. β-actin was used as an internal control. C: The expression of integrins α4 and β1 in HTR-8/SVneo trophoblast cells after siRNA HIF-1alpha treatment for 24 h. D: Number of invading cells in HTR-8/SVneo trophoblast cells between normoxia and hypoxia after integrin α4 siRNA treatment. The results, presented as means ± SEM, were produced by an ANOVA followed by Duncan's multiple comparison test with $P < 0.05$ as an indicator of significance.

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