Role of extracellular signal-regulated kinase and AKT cascades in regulating hypoxia-induced angiogenic factors produced by a trophoblast-derived cell line

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Abstract

During human pregnancy, trophoblasts play an important role in embryo implantation and placental development. Cytotrophoblast cells invade the uterine spiral arteries and differentiate into extravillous trophoblasts, resulting in the remodeling of the uterine vessels and fetoplacental vasculature. During early pregnancy, a physiologically hypoxic environment induces the production of angiogenic factors, such as vascular endothelial growth factor (VEGF), which are suggested to locally control the vascular remodeling. Endoglin, a cell-surface coreceptor for transforming growth factor-β1, is highly expressed in endothelial cells and syncytiotrophoblasts, and can be associated with endothelial nitric oxide synthase and vascular homeostasis. Several studies have recently suggested that some pregnancy-related complications, such as preeclampsia, have their origins early in pregnancy as a result of abnormalities in implantation and placental development. Although angiogenic factors are recognized as key molecules in placental development, little is known about the mechanism(s) of their regulation in trophoblasts. In this study, we elucidated the mechanisms underlying the regulation of VEGF and endoglin production under hypoxic conditions in the trophoblast-derived cell line, BeWo. We evaluated the role of the AKT–MTOR cascade and ERK kinase in the expression of VEGF and endoglin in response to hypoxia using various kinase inhibitors and small interfering RNA targeted against hypoxia-inducible factor (HIF)-1α (listed as *HIF1A* in Hugo Database). Our results suggest that both the phosphatidylinositol 3-kinase–AKT–MTOR–HIF-1α and ERK–HIF-1α signaling pathways are crucial for increasing VEGF and endoglin expression in response to hypoxia in BeWo cells.

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Introduction

During the first trimester of human pregnancy, extravillous trophoblasts from placental villi invade the deciduas and form plugs which temporarily occlude the spiral arteries and prevent maternal blood flow from entering the intervillous space, thereby creating a physiological low-oxygen environment (Jaffe *et al.* 1997, Burton *et al.* 1999). Rodesch *et al.* (1992) directly measured oxygen tension using a polarographic oxygen electrode during the first trimester of pregnancy. They demonstrated that the placenta develops in conditions of physiological hypoxia, in which the local oxygen concentration is as low as 1–2% during the first trimester. A significant increase was observed for placental PO_2 values measured at 12–13 weeks compared with those obtained at 8–10 weeks. More recently, Jauniaux *et al.* (2001) measured respiratory gases and acid–base gradients at 7–16 weeks of gestation. This report showed that before 11 weeks of gestation, the PO_2 level in the placenta was 2·5 times lower than that in deciduas (Jauniaux *et al.* 2001). Near the end of the first trimester, the trophoblast plugs progressively loosen, exposing the developing placenta to the maternal blood flow from ∼10 weeks of gestation (Jaffe & Woods 1993, Jauniaux *et al.* 2003). Therefore, the placenta initially develops under conditions of physiological hypoxia, and it is believed that the low-oxygen condition regulates placental development and extravillous trophoblast outgrowth (James *et al.* 2006).

A variety of angiogenic growth factors, including vascular endothelial growth factor (VEGF), are expressed in the placenta (Park *et al.* 1994, Cooper *et al.* 1995). VEGF is a powerful endothelial cell mitogen, and supports angiogenic remodeling of the early vessels, stimulating the formation of a capillary network to the placenta (Demir *et al.* 2004). In primary human endothelial cells, hypoxia increases the mRNA and protein levels of VEGF via hypoxia-inducible factor (HIF)-1α (listed as HIF1A in the Hugo Database) activation, which results in endothelial cell proliferation and tube formation (Yamakawa *et al.* 2003). VEGF has been implicated as playing an important role in placental angiogenesis (Ferrara *et al.* 2003, Zygmunt *et al.* 2003, Jauniaux *et al.* 2006).

Endoglin, a cell-surface coreceptor for transforming growth factor (TGF)-β1 and TGF-β3 isoforms, is highly

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expressed in endothelial cells and syncytiotrophoblasts (Cheifetz *et al*. 1992, Gougos *et al*. 1992, St-Jacques *et al*. 1994). Mutations in the gene encoding endoglin, *ENG*, are the underlying cause of hereditary hemorrhagic telangiectasia type 1, an autosomal dominant disorder characterized by arteriovenous malformations and focal loss of capillaries (McAllister *et al*. 1994). Endoglin is predominantly expressed in endothelial cells and is up-regulated by hypoxia (Brekken *et al*. 2002, Sanchez-Elsner *et al*. 2002, Duff *et al*. 2003). Recently, it was found that endoglin localizes to caveolae, where it can associate with endothelial nitric oxide synthase (eNOS) and regulate the activity and local tone of the vasculature (Toporsian *et al*. 2005). These data suggest the involvement of endoglin not only in cardiovascular development, but also in vascular homeostasis.

Although hypoxia caused by incomplete trophoblast invasion and impaired spiral arterial remodeling is thought to be a major cause of preeclampsia (PE) and intrauterine growth restriction (IUGR; Zhou *et al*. 1997, 2003, Damsky & Fisher 1998), how hypoxia affects placental development remains uncertain. Recent studies have implicated increased circulating soluble fms-like tyrosine kinase 1 (sFLT1, also known as soluble VEGF receptor 1) and soluble endoglin (sENG) as contributors to the pathogenesis of PE (Maynard *et al*. 2003, Levine *et al*. 2004, 2006, Venkatesha *et al*. 2006). The sFLT1 in the maternal circulation binds to VEGF and placental growth factor (PlGF), thereby preventing the action of these angiogenic growth factors on vascular tissues (Kendall *et al*. 1996, Maynard *et al*. 2003). Overexpression of *sFlt1* in rats leads to hypertension, proteinuria, and glomerular endotheliosis, the classical manifestations of PE, suggesting that excess circulating sFLT1 may have a causal role in PE (Maynard *et al*. 2003). sENG has been shown to exert antiangiogenic properties, possibly via an impairment of TGF-β1 signaling in the vasculature (Toporsian *et al*. 2005, Bernabeu *et al*. 2007). Moreover, sENG inhibits the formation of capillary tubes, and protein cooperates with sFLT1 to induce endothelial dysfunction *in vitro*, and a severe PE-like illness *in vivo* (Venkatesha *et al*. 2006). These findings suggest that both VEGF and endoglin may be crucial factors that must be strictly regulated to achieve normal placental development.

Little is known regarding the mechanisms that regulate VEGF and endoglin expression in the placenta; therefore, we elucidated the roles of the AKT–MTOR and ERK cascades in the regulation of VEGF and endoglin production under hypoxic conditions in the trophoblast-derived cell line, BeWo.

Materials and Methods

Materials

The phosphatidylinositol 3-kinase (PI3K) inhibitor, LY294002, and MAPK kinase (MEK) inhibitor, PD98059, were purchased from Calbiochem (San Diego, CA, USA). The MTOR inhibitor, rapamycin, was purchased from Sigma Chemical Co. Anti-ERK polyclonal antibodies, anti-phosphorylated ERK polyclonal antibodies, anti-AKT polyclonal antibodies, anti-phosphorylated AKT polyclonal antibodies, and anti-phosphorylated MTOR antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). The anti-HIF-1α monoclonal and anti-NFκB antibodies were obtained from Becton Dickinson (Franklin Lakes, NJ, USA).

Cell culture

The BeWo choriocarcinoma cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). BeWo cells were cultured at 37 °C in DMEM/F-12 with 10% FBS in a water-saturated atmosphere of 95% O_2 and 5% CO_2, unless otherwise indicated. Prior to treatment, BeWo cells were serum-starved in DMEM/F-12 medium containing 0·5% BSA for 16 h. Hypoxic exposure was carried out under 1% O_2, 5% CO_2, and 94% N_2 in a modular incubator (Hirasawa, Tokyo, Japan) for the times indicated in the figures (2–6 h). Inhibitors were added 1 h before hypoxia.

Western blot analysis

The BeWo cells were starved and incubated under hypoxic conditions for the times indicated in the figures. Cells were then washed twice with ice-cold PBS, lysed, and separated into cytoplasmic and nuclear fractions using a Nuclear Extract Kit according to the manufacturer's protocol (Active Motif, Carlsbad, CA, USA). The protein concentrations of the supernatants were determined using the Bio-Rad protein assay reagent. Equal amounts of proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Blocking was done in 10% BSA in 1× Tris-buffered saline. Western blot analyses were performed with various specific primary antibodies. Immunoreacted bands in the immunoblots were visualized with HRP-coupled goat anti-rabbit or anti-mouse immunoglobulin by using the enhanced chemiluminescence western blotting detection system.

Real-time PCR

Total RNA was isolated using a TRIzol-based approach, according to the manufacturer's protocol (Invitrogen), and the RNA was reverse-transcribed using the first-strand cDNA synthesis kit (Amersham Biosciences) as recommended. Real-time PCR was carried out using a Roche LightCycler 2.0 system (Roche Diagnostics). The synthesized cDNA was diluted to 20 ng/μl and used at 50 ng per reaction. The Taqman Master kit, in combination with the Universal Probe Library (Human), was used to assess gene expression (Roche Diagnostics). PCR primers for Taqman/Probe Library assays were designed using the Probe Library Assay Design Center (https://www.roche-applied-science.com/sis/rtpcr/upl/acenter.jsp?id=030000), and included the following: *VEGF*-specific primers 5′-TTG AGT TAA

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ACG AAC G-3′ (forward) and 5′-GGT TCC CGA ACC CTG AG-3′ (reverse); endoglin primers 5′-CCA CTG CAC TTG GCC TAC A-3′ (forward) and 5′-GCC CAC TCA AGG ATC TGG-3′ (reverse); *HIF-1α* primers 5′-TTT TTC AAG CAG TAG GAA TTG GA-3′ (forward) and 5′-GTG ATG TAG TAG CTG CAT GAT CG-3′ (reverse); and *GAPDH* primers 5′-AGC CAC ATC GCT CAG ACA-3′ (forward) and 5′-GCC CAA TAC GAC CAA ATC C-3′ (reverse). Quantitative real-time PCR was performed using a LightCycler 2.0, and the data were analyzed by the LightCycler Software program 4.05 (Roche Diagnostics) using a calibrator-normalized relative quantification approach. Relative quantification was based on GAPDH expression.

Small interfering RNA expression

The small interfering RNA (siRNA) against HIF-1α (stealth RNAi) was custom synthesized (Invitrogen). Primer sequences were as follows: sense, 5′-GAG GAA ACU UCU GGA UGC UGG UGA T-3′; antisense, 5′-AUC ACC AGC AUC CAG AAG UUU CCU C-3′. Sequences of the stealth RNAi negative control (scrambled siRNA) were as follows: sense, 5′-GAG AAU CCU GUA GGU UCG GUA GGA U-3′; antisense, 5′-AUC CUA CCG AAC CUA CAG GAU UCU C-3′. The siRNA and scrambled siRNA were transiently transfected for 24 h using LipofectAMINE Plus (Invitrogen), according to the manufacturer's protocol. Briefly, 50% confluent BeWo cells were seeded and incubated overnight. For the transfection of each sample, oligomer-LipofectAMINE Plus complexes were prepared as follows: 100 pmol of siRNA oligomer were diluted in 250 μl of Opti-MEM (Invitrogen). LipofectAMINE Plus was mixed gently before use, and then a 5-μl aliquot was diluted in 250 μl of Opti-MEM, mixed gently, and incubated for 5 min at room temperature. After the 5-min incubation, the diluted oligomer was combined with the diluted LipofectAMINE Plus, mixed gently, and incubated for 20 min at room temperature. The oligomer-LipofectAMINE Plus complexes were added to each well containing cells and medium, and mixed gently by rocking the plate back and forth. The cells were incubated at 37 °C in a CO_2 incubator for 24 h, and then cells were prepared for each assay.

ELISA

The cell culture medium from cells grown under various conditions was collected and used to determine the secretion of VEGF and endoglin using an ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were carried out according to the manufacturer's protocol. Briefly, BeWo cells were cross-linked with 1% HCHO for 10 min. Cell extracts were sonicated to sheer chromatin to an average size of ∼600 kb. The extract was divided into aliquots, and antibodies were added to the aliquots at a 1:100 dilution for immunoprecipitation. An anti-rabbit IgG antibody was used as a negative control. After immunoprecipitation, an aliquot of each captured immunocomplex was subjected to western analysis to confirm that the captured chromatin contained the transcriptional co-regulator corresponding to the specific antibody that had been used for ChIP. For the remainder of the sample, cross-links in the immunoprecipitated chromatin were reversed by heating with proteinase K at 65 °C overnight, and then DNA was purified using a MinElute Reaction Cleanup kit (Qiagen) and resuspended in 10 μl of 1× TE. The purified ChIP-caputured DNA was analyzed by PCR. PCR amplifications were performed with the following specific primer pair for *VEGF*: 5′-AAG ACA TCT GGC GGA AAC C-3′ (forward) and 5′-ACA ATT GGT CGC TAA CCG AG-3′ (reverse). The PCR products were separated by electrophoresis on a 2% agarose gel.

Statistical analysis

Statistical analysis was performed using one-way ANOVA followed by a Tukey's *post hoc* test, and $P<0{\cdot}05$ was considered to be significant. Data in figures are presented as the means ± S.E.M.

Results

Regulation of VEGF and endoglin by hypoxic stimuli

To investigate the trophoblast response to a hypoxic stimulus, serum-starved BeWo cells were incubated under hypoxic conditions (1% oxygen) for various times. Real-time PCR assays were performed to examine whether hypoxia up-regulated the expression of *VEGF* and endoglin. We observed that *VEGF* mRNA expression was increased 1·52±0·38-, 4·05±0·27-, and 3·13±0·35-fold at 2, 4, and 6 h respectively compared with cells incubated under normoxia (Fig. 1A). The endoglin mRNA expression increased 3·64±0·52- and 1·51±0·33-fold at 4 and 6 h respectively in comparison to the cells incubated under normoxia (Fig. 1B).

Differential activation of AKT and ERK cascades by hypoxia

We next examined whether hypoxia activates the AKT and ERK cascades in the BeWo cells. The cells were incubated under hypoxic conditions (1% oxygen) for various times, and then the lysates were analyzed by western blotting with an anti-phospho-AKT, -AKT, -phospho-MTOR, or -MTOR antibody. Although hypoxia did not affect the expression of AKT (Fig. 2A, lower panel), it induced a transient phosphorylation of AKT lasting 6–10 h, followed by a decrease in AKT phosphorylation (Fig. 2A, upper panel). Hypoxia also transiently increased the phosphorylation of the

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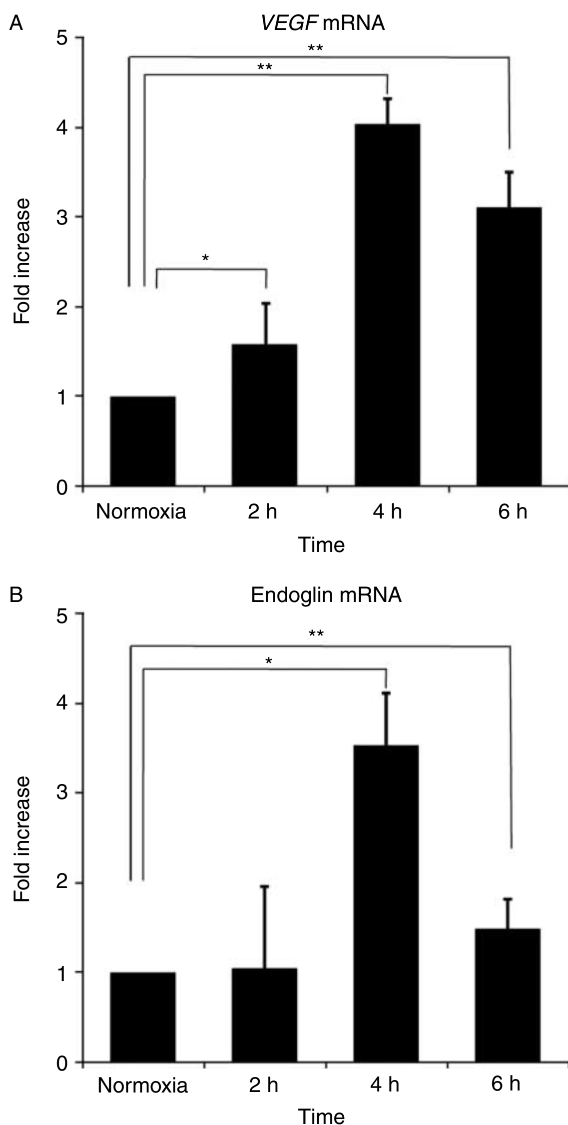

Figure 1 Effect of hypoxia on *VEGF* and endoglin mRNA expression in BeWo cells. BeWo cells were cultured in serum-free medium for 16 h and incubated under 1% O_2 for various times (indicated in the figure). Total RNA was isolated and reverse-transcribed, and then the resulting cDNA was used for real-time PCR to assess the mRNA expression of *VEGF* (A) and endoglin (B) relative to *GAPDH*. Values shown represent the means ± S.E.M. from at least three separate experiments. Significant differences are indicated by asterisks. $^{**}P<0{\cdot}01$ and $^{*}P<0{\cdot}05$.

substrate of AKT, MTOR, at 10 h in the BeWo cells (Fig. 2B, upper panel). Although hypoxia did not affect the expression of ERK (Fig. 2C, lower panel), the phosphorylation of ERK occurred at 6 h under hypoxic conditions, reached a plateau at 10 h, and was sustained until 24 h in the BeWo cells (Fig. 2C, upper panel). These results demonstrate that hypoxia induces the phosphorylation of the AKT–MTOR cascade and ERK with different time courses of activation.

AKT- and ERK-dependent expression of VEGF and endoglin induced by hypoxia

We observed that increased expression of *VEGF* and endoglin occurs under hypoxic conditions; however, the mechanisms underlying the hypoxia-induced increase in *VEGF* and endoglin expression were still unclear. Therefore, we investigated the AKT- and ERK-dependent expression of *VEGF* and endoglin using kinase inhibitors. Cells were treated for 1 h with vehicle (DMSO), a PI3K inhibitor LY294002 (LY), the MTOR inhibitor rapamycin (Rapa), or the ERK inhibitor PD98059 (PD), and then exposed to hypoxia for 4 h. Total RNA was isolated and reverse-transcribed, and cDNA was used for real-time PCR. Whereas the vehicle (DMSO) had no effect on *VEGF* or endoglin mRNA expression, pretreatment with LY294002, rapamycin, and PD98059 attenuated the hypoxia-induced expression of both *VEGF* and endoglin mRNA (Fig. 3A and B). We next confirmed that the AKT and ERK kinases were involved in the expression of secreted VEGF and endoglin proteins using ELISA. Pretreatment with either LY294002, rapamycin, or PD98059 attenuated the hypoxia-induced increase in VEGF and endoglin secretion (Fig. 3C and D).

Hypoxia recruits HIF-1α to the promoters of VEGF and endoglin

To examine whether hypoxia induces HIF-1α translocation into the nucleus, cells were incubated under hypoxic conditions (1% oxygen) for various times, and then nuclear fractions and whole cell lysates were prepared for analysis by western blotting. *HIF-1α* expression was increased in the nuclear fraction at 6 and 10 h under hypoxic conditions (Fig. 4A, upper panel). However, the expression of *HIF-1β* did not differ among the lanes in the nuclear fraction (Fig. 4A, middle panel), suggesting that hypoxia led to the translocation of HIF-1α into the nucleus, but did not alter the expression of *HIF-1β*. We next examined whether hypoxia enhances the binding of HIF-1α to the promoters for VEGF and endoglin. The BeWo cells were incubated under hypoxic conditions (1% oxygen) for various times, and then used to prepare lysates that were subjected to ChIP with an antibody against HIF-1α. The ChIP-captured DNA was subjected to PCR amplification using PCR primers located downstream and upstream of the *HIF-1α*-binding sites on the promoters for *VEGF* (Fig. 4B, upper panel) and endoglin (Fig. 4C, upper panel). The results indicated that hypoxia induced the translocation of *HIF-1α* into the nucleus, and also increased the binding of *HIF-1α* to the promoters of both *VEGF* and endoglin in these cells.

Effect of HIF-1α silencing on the expression of VEGF and endoglin induced by hypoxia

To confirm that *HIF-1α* was required for the induction of *VEGF* and endoglin by hypoxia, we examined the effects of silencing *HIF-1α* on the hypoxia-induced expression of

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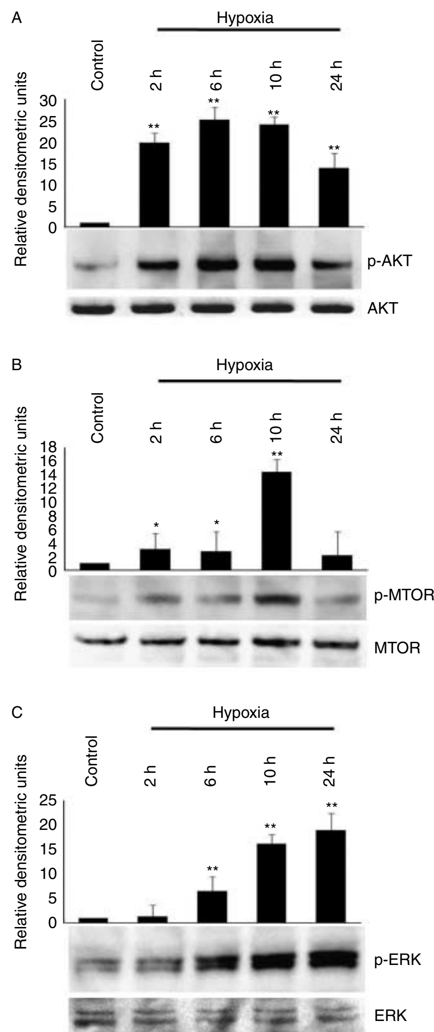

Figure 2 Hypoxia increases AKT, MTOR, and ERK phosphorylation in BeWo cells. BeWo cells were exposed to hypoxia for 0 (control) to 24 h, and samples from the cytoplasmic fraction were subjected to western blotting for (A) phospho-AKT (upper panel) and AKT (lower panel), (B) phospho-MTOR (upper panel) and MTOR (lower panel), and (C) phospho-ERK (upper panel) and ERK (lower panel), with the density of the control bands set arbitararily at 1·0. Values shown represent the means ± S.E.M. from at least three separate experiments. Significant differences are indicated by asterisks. $^{**}P<0{\cdot}01$ and $^{*}P<0{\cdot}05$.

VEGF and endoglin mRNA. Demonstrating that the siRNA was effective, the expression of *HIF-1α* in BeWo cells transfected with the siRNA against HIF-1α was found to be significantly lower than that in the BeWo cells transfected with the scrambled siRNA (Fig. 5A, upper panel). The specificity of the siRNA is illustrated by the equal expression of β-actin in cells transfected with both the specific and scrambled siRNAs (Fig. 5A, middle panel). We further confirmed the *HIF-1α* silencing using quantitative real-time PCR. The expression of *HIF-1α* was decreased 0·3 times in the BeWo cells transfected with siRNA (Fig. 5B). The *VEGF* mRNA expression induced by hypoxia was increased 3·23 ± 0·19-fold in the BeWo cells transfected with scrambled siRNA; transfection with *HIF-1α* siRNA significantly attenuated the *VEGF* mRNA expression induced by hypoxia (Fig. 5C). Similarly, while the expression of endoglin mRNA induced by hypoxia was increased 2·31 ± 0·35-fold in BeWo cells transfected with the scrambled siRNA, transfection with *HIF-1α* siRNA significantly attenuated the expression of endoglin mRNA (Fig. 5D). These results suggest that HIF-1α is a necessary determinant of the hypoxia-induced expression of VEGF and endoglin in trophoblast-derived BeWo cells.

Discussion

Our study uncovered the signaling mechanism responsible for the hypoxia-induced release of angiogenic factors in BeWo cells. We have therefore demonstrated two major findings: i) hypoxia (1% oxygen) differentially induces both the mRNA expression and protein secretion of VEGF and endoglin via the AKT–MTOR and ERK signaling cascades and ii) *HIF-1α* is a major inducer of the expression of both *VEGF* and endoglin.

In early pregnancy, trophoblasts grow and develop to form the placenta under physiological hypoxia, where the various angiogenic factors are dramatically up-regulated. In PE, the trophoblasts fail to properly migrate and transform into a normal placenta (Zhou *et al.* 1997, 2003, Damsky & Fisher 1998). These facts led us to examine the molecular mechanisms underlying the regulation of angiogenic factors in trophoblast cells. However, primary cultured trophoblasts are heterogeneous and are not suitable for the experiments performed in this study, such as the transfection of siRNA. Therefore, in our study, we used trophoblast-derived human BeWo cells as a trophoblast model. These cells are similar in morphology to primary trophoblast cultures, and they are well established as an *in vitro* model to study trophoblast development and function (Ellinger *et al.* 1999, Heaton *et al.* 2008, Neelima & Rao 2008).

Normally, trophoblast cells transform from an epithelial phenotype to an endothelial phenotype as they invade the maternal deciduas and myometrium in a process termed pseudovasculogenesis. Migrating trophoblasts transform the maternal spiral arterioles that supply maternal blood to the placenta from small caliber resistance vessels to large caliber capacitance vessels, allowing adequate maternal blood flow to the placenta (Starzyk *et al.* 1997, Lyall 2005). The epidermal growth factor was reported to induce syncytialization of cytotrophoblasts and the secretion of human chorionic gonadotropin and human placental lactogen *in vitro* (Morrish *et al.* 1987). Colony-stimulating factor (CSF), granulocyte–macrophage CSF (GM-CSF), a TGF-β superfamily member, as well as VEGF, have been described to promote the syncytialization of trophoblasts (Garcia-Lloret *et al.* 1994, Crocker *et al.* 2001, Yang *et al.* 2003*a*, Li *et al.* 2005).

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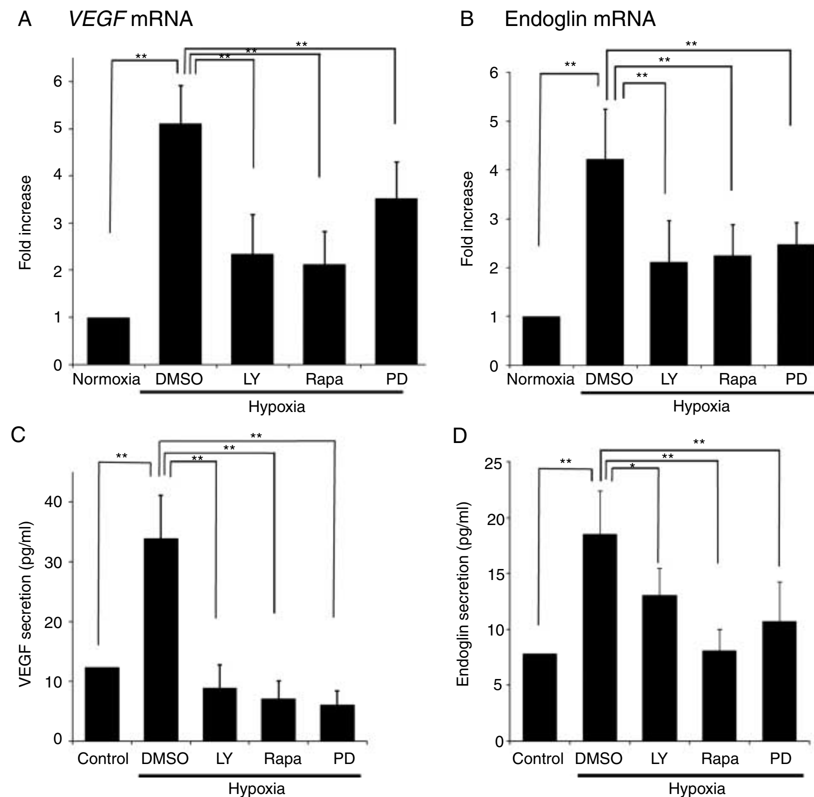

Figure 3 Role of AKT and ERK kinases on hypoxia-induced *VEGF* and endoglin mRNA in BeWo cells. BeWo cells were incubated in serum-free medium for 16 h and then for 30 min in the presence or in the absence of either 50 nM LY294002 (LY), 50 nM rapamycin (Rapa), 50 nM PD98059 (PD), or 50 nM LY +50 nM PD prior to incubation in a 1% O_2 hypoxic chamber for 4 h. Total RNA was isolated and reverse-transcribed, and then the resulting cDNA was used for real-time PCR to measure the mRNA expression of *VEGF* (A) and endoglin (B) relative to GAPDH. Cell culture media were collected and used to determine the level of secreted VEGF (C) and endoglin (D) using ELISA kits. Values shown represent the means ± S.E.M. from at least three separate experiments. Significant differences are indicated by asterisks. $^{**}P<0·01$; $^{*}P<0·05$.

Our current results show that a hypoxic environment induces both *VEGF* and endoglin expression (Fig. 1) in a trophoblast-derived cell line. However, the role of physiological hypoxia in early pregnancy and placental development is unclear, and the signaling mechanisms regulating the hypoxia-induced expression of angiogenic factors have not been fully investigated.

Our study demonstrates that AKT–MTOR activation is crucial for the production of angiogenic factors under hypoxic conditions in BeWo trophoblast-derived cells (Fig. 3). Recent studies have shown that PI3K and AKT play an important role in regulating tumor growth and angiogenesis through the upregulation of *VEGF* and *HIF-1* expression. The central role of AKT signaling in placental growth regulation was confirmed in *AKT1*-null mice, which display IUGR (Yang *et al*. 2003*b*, Yung *et al*. 2008). Together, these studies demonstrated that the inactivation of AKT caused hypotrophy and structural abnormalities of the placenta that likely contributed to placental insufficiency and subsequent impairment of fetal growth.

Of interest, inhibition of PI3K/AKT/MTOR signaling in endothelial cells by rapamycin reverses the pathological effects associated with excess VEGF signaling in the tumor vasculature by either reducing AKT activity or blocking MTOR (Phung *et al*. 2006). It has been demonstrated that MTOR, which is a substrate of AKT, is essential for the growth and proliferation of early mouse embryonic stem cells (Gangloff *et al*. 2004, Murakami *et al*. 2004). Embryos that are MTOR deficient die shortly after implantation as a result of impaired cell proliferation in both the embryonic and extra-embryonic compartments. These findings suggest that MTOR may play an important role in controlling trophoblast cell growth and proliferation (Wen *et al*. 2005). In order to investigate the physiological roles of VEGF and endoglin secreted from trophoblasts, co-culture methods with endothelial progenitor cells will be needed, and the morphological

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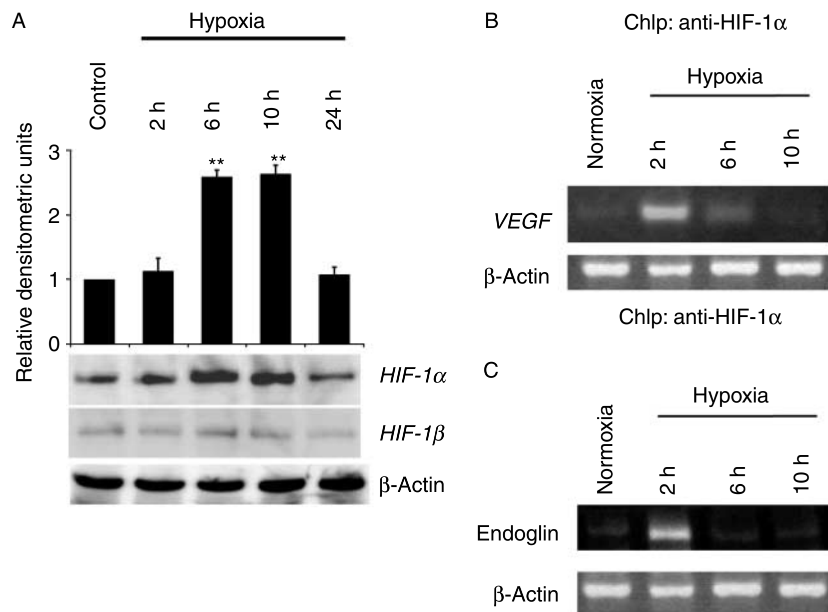

Figure 4 *HIF-1α/β* is translocated into the nucleus and recruited to the VEGF promoter by hypoxia. (A) BeWo cells were exposed to 1% O_2 for 0 (control) to 24 h, and samples from nuclear fractions were subjected to western blotting for *HIF-1α* (upper panel) and *HIF-1β* (middle panel). Whole cell lysates were also evaluated for HIF expression, and the level of β-actin was detected as an internal loading control (lower panel). Relative densitometric units of the HIF-1α bands are shown in the top panel, with the density of the control bands set arbitararily at 1·0. Values shown represent the means ± S.E.M. from at least three separate experiments. Significant differences are indicated by asterisks (**$P<0·01$). BeWo cells were exposed to 1% O_2 for 0 (control) to 10 h, and lysates were chromatin immunoprecipitated with an antibody against HIF-1α. (B) The chromatin immunoprecipitation-captured DNA was subjected to PCR amplification using PCR primers located downstream and upstream of the HRE site of the promoter for *VEGF* (upper panel). PCRs using primers for β-actin were carried out using total cell extracts as an internal control. (C) The chromatin immunoprecipitation-captured DNA was subjected to PCR amplification using PCR primers located downstream and upstream of the HRE site of the promoter for endoglin (upper panel). PCRs using primers for β-actin were carried out using total cell extracts as an internal control (lower panel).

changes in BeWo or other trophoblast cells should be addressed by detecting differentiation, as well as examining migration and invasion.

The present study provides evidence that hypoxia induces the expression of angiogenic factors such as *VEGF* and endoglin via the AKT and ERK cascades, thus leading to the transcriptional activation of *HIF-1α* in BeWo cells. *HIF-1α* is a transcription factor which is activated by hypoxia, and is involved in the adaptative response of cells to oxygen deprivation. During hypoxic stress, *HIF-1α* triggers the overexpression of genes coding for glycolytic enzymes and angiogenic factors (Diaz-Gonzalez *et al.* 2005). *HIF-1α* is a substrate for various kinase pathways, including PI3K and the MAP kinases, ERK and p38 (Minet *et al.* 2001). Previous studies have demonstrated that the activation of AKT signaling in endothelial cells results in vessel relaxation via increases in eNOS activity (Luo *et al.* 2000, Hisamoto *et al.* 2001*a*,*b*, Northcott *et al.* 2002). Hypoxia was also shown to regulate eNOS activity and NO production via AKT activation in porcine coronary artery endothelial cells (Chen & Meyrick 2004). On the other hand, hypoxia increases the phosphorylation of ERK, followed by the stabilization and activation of *HIF-1α* which enhances *HIF-1α*-dependent transcriptional activation of *VEGF* in hamster fibroblasts (Berra *et al.* 2000). Although siRNA targeted against *HIF-1α* significantly blocked the secretion of VEGF and endoglin in our study, the involvement of other transcription factors should be investigated to obtain a better understanding of the full signaling pathway(s).

During PE, the fetal trophoblasts fail to properly invade the maternal myometrium and spiral arterioles (Meekins *et al.* 1994). The mechanisms underlying normal and failed trophoblast invasion are still poorly understood. In PE, major uteroplacental pathology is characterized by the coexistence of poor arterial remodeling (Pijnenborg *et al.* 1991) and minimal invasion of the deciduas and its vessels by extravillous trophoblast cells, which also fail to develop a vascular endothelial phenotype (Zhou *et al.* 1997). There is no

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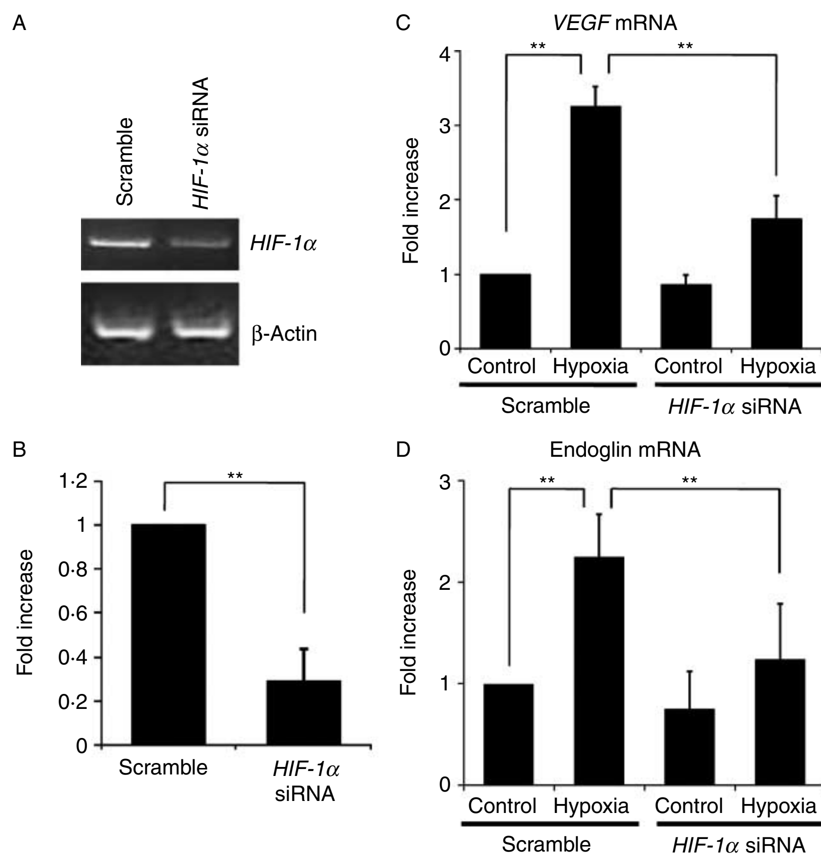

Figure 5 Effect of *HIF-1α* silencing on hypoxia-induced expression of *VEGF* and endoglin mRNA. BeWo cells were transfected with scrambled or *HIF-1α*-specific siRNA as described in the Materials and Methods section. RNA was extracted from the cells, and RT-PCR assays were performed to detect the mRNA expression of *HIF-1α* (A, upper panel) and β-actin (A, lower panel). Quantitative real-time PCR assays were performed to measure *HIF-1α* (B) relative to *GAPDH*. Values shown represent the means ± S.E.M. from at least three separate experiments. Significant differences are indicated by asterisks (**$P<0{\cdot}01$). To determine the effect of *HIF-1α* silencing on hypoxia-induced *VEGF* and endoglin mRNA expression, BeWo cells were transfected with scrambled or *HIF-1α*-specific siRNA as described in the Materials and Methods section. Total RNA was collected and reverse-transcribed, and then the resulting cDNA was used for real-time PCR to measure the mRNA expression of *VEGF* (C) and endoglin (D) relative to GAPDH. Values shown represent the means ± S.E.M. from at least three separate experiments. Significant differences are indicated by asterisks (**$P<0{\cdot}01$).

ideal model for human placentation, because the human placenta is unique compared to that of most other mammals. However, the linkage between placental hypoxia and maternal vascular dysfunction has been proposed to occur via placental syncytiotrophoblast basement membranes shed by the placenta or via placental secretion of angiogenic factors such as sFlt1 and endoglin that bind VEGF and PlGF in the maternal circulation. Therefore, the mechanisms that initiate PE in humans have been elusive, but some parts of the puzzle have begun to come together. In addition, although there have been several reports suggesting that hypoxia induces the expression of endoglin in PE (Levine *et al.* 2006, Venkatesha *et al.* 2006), the details about this mechanism remain uncertain. Therefore, further investigations will be necessary to clarify the physiological roles of VEGF and endoglin secreted from trophoblasts. In addition, *in vivo* studies are expected to provide insight into the importance of these pathways in placental formation and their role in PE.

In summary, the present study provides the first evidence that both the AKT/MTOR and ERK signaling pathways are involved in hypoxia-induced expression of both VEGF and endoglin in trophoblast-derived BeWo cells. Although no *in vitro* model provides a perfect approach for directly examining early placentation or the pathogenesis of PE, our findings show that these mechanisms are likely to be necessary for placental development in early pregnancy, and may also have important implications for understanding the pathogenesis of PE.

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Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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