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Low oxygen tension induces Krüppel-Like Factor 6 expression in trophoblast cells

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ABSTRACT

The transcription factor Krüppel-Like Factor 6 (KLF6) has important roles in cell differentiation, angiogenesis, apoptosis, and proliferation. Furthermore, there is evidence that KLF6 is required for proper placental development. While oxygen is a critical mediator of trophoblast differentiation and function, the involvement of oxygen in the regulation of KLF6 expression remains unexplored. In the present study we examined the expression of KLF6 in placental tissue from uncomplicated and preeclamptic pregnancies, the latter often characterized by an inadequately perfused placenta. We also determined the effect of hypoxia and the involvement of Hypoxia-Inducible Factor 1α (HIF-1α) on the expression of KLF6 in cultured trophoblast cells and placental tissues. Results revealed that villous, interstitial and endovascular extravillous cytotrophoblasts from placentas from normal and preeclamptic pregnancies express KLF6. In addition, KLF6 immunoreactivity was higher in the placental bed of preeclamptic pregnancies than in those of uncomplicated pregnancies. We demonstrated that hypoxia induced an early and transient increase in KLF6 protein levels in HTR8/SVneo extravillous cytotrophoblast cells and in placental explants. Reoxygenation returned KLF6 protein to basal levels. Moreover, hypoxia-induced up-regulation of KLF6 expression was dependent on HIF-1α as revealed by siRNA knockdown in HTR8/SVneo cells. These results indicate that KLF6 may mediate some of the effects of hypoxia in placental development. The regulation of KLF6 protein levels by oxygen has significant implications for understanding its putative role in diseases affected by tissue hypoxia.

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1. Introduction

Oxygen tension is a key factor in the regulation of cytotrophoblast differentiation, placental development, and trophoblast invasion in uncomplicated and pathological pregnancies [1–4]. Early placentation occurs in an environment characterized by low oxygen tension, which promotes invasion of the maternal spiral arteries by extravillous cytotrophoblasts and the differentiation of these cells into endothelial-like cells. The invasion of the spiral arteries by extravillous cytotrophoblasts transforms these vessels into high-calibre, high-capacitance conduits capable of providing adequate placental perfusion to sustain the growing fetus. Between 10 and 12 weeks of gestation, intervillous blood flow increases and the trophoblast is exposed to a marked rise in oxygen levels [5].

Altered placental oxygenation has been linked to shallow trophoblast invasion and the development of preeclampsia. Preeclampsia is a complex pregnancy-specific disorder characterized by a systemic maternal inflammatory response associated with endothelial dysfunction, hypertension, and proteinuria [6]. Multiple pathogenic mechanisms have been implicated in this disorder, including hypoxia and hypoxia/re-oxygenation (H/R) injury [5,7]. Deficient trophoblast invasion and incomplete remodelling of the spiral arteries lead to further intermittent placental perfusion with subsequent H/R stress [6,8].

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Trophoblasts, as all higher eukaryotic cells, respond to low oxygen tension with a series of adaptive modifications in gene expression. Hypoxia Inducible Factors (HIFs) play a central role in oxygen homeostasis. Under normoxia, HIF-α subunits (HIF-1α, HIF-2α, and HIF-3α) are hydroxylated by prolyl hydroxylases, and targeted for proteasomal degradation. In the absence of oxygen HIF-α subunits achieve stability, translocate to the nucleus, and heterodimerize with ARNT/HIF-1β. The dimer binds to hypoxia-response elements present in target genes regulating functions such as angiogenesis, metabolism, invasion, metastasis, and apoptosis [9].

Transcription factors have important functions in the adaptive response of cells and organisms to stress conditions [10,11]. KLF6 is a member of the Specificity protein 1/Krüppel-like transcription factor family (Sp1/KLF), initially cloned from human placenta [12], leukocytes [13], and liver [14]. Several external and internal signals have been reported to stimulate KLF6 expression with the subsequent transcriptional regulation of diverse target genes implicated in tissue remodelling, angiogenesis, vasculogenesis, proliferation, apoptosis, and differentiation [15–19]. Although KLF6 is a ubiquitous transcription factor, its highest expression level is found in the placenta [12] and *Klf6*$^{-/-}$ knockout mice die by embryonic day 12.5 with a phenotype characterized by impaired placental development, poorly defined liver, and disorganized yolk sac vascularization [20]. However, KLF6 relevance in the placenta has not been fully studied. We have previously demonstrated that KLF6 is present in the nucleus and cytoplasm of villous trophoblasts in a regulated fashion during syncytialization. In addition, KLF6 contributes to the transcriptional activation of human chorionic gonadotropin β5, pregnancy specific glycoprotein (PSG) 5 and PSG3 [21]. Furthermore, KLF6 is required for proper trophoblast cell fusion since KLF6 knockdown impairs cell–cell fusion and decreases the expression of the fusogenic protein syncytin-1 and the cell cycle inhibitor p21$^{Cip1/Waf1}$ [19]. However, the expression of KLF6 in extravillous cytotrophoblast as well as its expression under hypoxic conditions remain unexplored.

Here, we examined KLF6 expression in the maternal-fetal interface of normal term as well as preeclamptic pregnancies, and analyzed whether changes in oxygen tension influence the expression of KLF6 in an extravillous cytotrophoblast cell model and in human normal term placental explants. In addition, we explored the dependence of KLF6 expression on HIF-1α.

2. Materials and methods

2.1. Placental samples

Placentas for immunohistochemistry were collected from preeclamptic patients and healthy pregnant women with uncomplicated pregnancies. Paraffin blocks of formalin-fixed placental samples from five preeclamptic women were selected from the archives of the Department of Pathology at Medical Sciences School, State University of Rio de Janeiro (Rio de Janeiro - RJ, Brazil). They included maternal-fetal interface areas from each placenta (from 34 to 37 weeks of gestation) obtained immediately after caesarean delivery. Pregnancies were conventionally diagnosed as preeclampsia by increased blood pressure (≥140 mmHg systolic or ≥90 mmHg diastolic on ≥2 occasions at least six hours apart) occurring after the 24th week of gestation in a pre-normotensive woman, accompanied by proteinuria (≥0.3 g/24 h). For each case, one block was selected for immunohistochemical staining. Control (normotensive) cases consisted of eight third-trimester placentas from elective caesarean deliveries at 39–40 weeks of gestation from healthy mothers and fetuses (without pregnancy complications or previous diseases) collected at the Hospital of the University of São Paulo (SP-SP, Brazil) within 30 min of delivery. Three random tissue samples (~10 mm thick each) were taken from each placenta and fixed in 4% paraformaldehyde for 12–18 h prior to embedding in paraffin for serial sectioning. Informed consent for the use of placental tissues was obtained at the time of delivery. The study was approved by the Ethics Committee for Human Research at the School of Medicine, University of São Paulo.

2.2. Placental explant cultures

Tissues from normal term placentas were obtained after caesarean delivery and processed within 30 min of delivery. These placentas were obtained from unidentified anonymous patients with the approval of the local Advisory Committee of Biomedical Research in Humans, Córdoba, Argentina. Villous tissue free of visible infarcts, calcification or haematoma was sampled from the maternal-fetal interface, cut into small pieces, and washed with 154 mmol/L NaCl to remove blood. Tissue samples were further cut and washed thoroughly to obtain pieces of very small size (1–1.5 cubic millimeters) free of calcifications, infarctions, clots, fibrosis and visible vasculature.

Subsequently, explants were placed in 24-well plates at a ratio of three explants per well in 1 mL of DMEM-F12 (Invitrogen) supplemented with 10% v/v fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin/0.1 mg/mL streptomycin), and incubated at 37 °C and 5% CO_2. For incubations in hypoxia, explants were cultured in a humidified chamber in an atmosphere of a mixture of gases (1.3 ± 0.1% oxygen and 5.0 ± 0.2% CO_2) balanced with N_2 at 37 °C.

2.3. Culture of HTR8/SVneo trophoblast cells

The human immortalized first trimester trophoblast cell line HTR8/SVneo [22] was cultured in RPMI 1640 medium (Invitrogen) supplemented with gentamicin 0.05 mg/mL (Schering-Plough) and 10% v/v FBS. For incubations in standard conditions (20% oxygen), cells were placed in a CO_2 incubator. For incubations in hypoxia (0.2% oxygen), cells were placed in a chamber that was flushed with a gas mixture of 5%CO_2/95% N_2. Oxygen concentrations within the chamber were maintained at 0.2% by means of a ProOx 110 oxygen regulator (Biospherix Inc., Lacona, NY).

2.4. Immunohistochemistry

Trilogy IHC pre-treatment Solution (Sigma-Aldrich, St. Louis, MO) was used for deparaffinization, rehydration, and antigen retrieval simultaneously according to the manufacturer's instructions. The slides were then sequentially incubated with blocking reagent (Reveal TM Biotin-free Polyvalent HPR, SPB-999, Spring Bioscience Corp, Pleasanton, CA) and mouse monoclonal anti-cytokeratin (LMW-AE1, Cell Marque) or mouse monoclonal anti-KLF6 (clone 2c11; its specificity previously determined [23]), diluted at 1:350 and 1:500 in phosphate-buffered saline, respectively (one hour at room temperature). Control samples received mouse non-immune serum diluted 1:500 in phosphate-buffered saline. The samples were incubated with the rabbit anti-mouse secondary antibody and then with the goat anti-rabbit secondary antibody conjugated to horseradish peroxidase for 15 min, and revealed with 3,3′-diaminobenzidine (Sigma-Aldrich, 1:50 v/v) in Reveal TM specific reagent (Reveal TM Biotin-free Polyvalent HPR, SPB-999, Spring Bioscience Corp, Pleasanton, CA). After an additional washing, the slides were counterstained with Mayer's Hematoxylin. Images were taken on an Axioskop 2 Optical Microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany) connected to a computer running Axio Vision 4.7 (Carl Zeiss MicroImaging GmbH) processing software.

Five images from each slide (from three different paraffin blocks, n = 15) were randomly selected and captured using a 10× objective, 1388× 1040 pixels and a resolution of 4 pixels/μm^2. KLF6 immunoreactivity was measured using computer-assisted image analysis (ImageJ, NIH, USA) in a microscopic area calculated as 90.220 μm^2. The results were recorded as pixels/μm^2 and the means in each group statistically compared using Student's t-test. Results were expressed as mean ± SD.

2.5. SDS-PAGE and western blotting

Post treatment, protein extracts were prepared from placental explants and HTR8/SVneo cells avoiding oxygenation to prevent HIF-1α protein degradation. Protein samples were loaded onto a 10% SDS-PAGE gel. After electrophoresis, proteins were electro-transferred to nitrocellulose (Amersham Bioscience). Membranes were blocked in Tris-buffered saline (TBS: 20 mM Tris−HCl, 150 mM NaCl pH 7.8) supplemented with 0.2% v/v Tween-20 (TBS-T) containing 5% w/v non-fat milk for one hour at room temperature with shaking. Blots were incubated with primary antibodies diluted in 5% w/v non-fat milk in TBS-T overnight at 4 °C. The following antibodies were used: mouse monoclonal anti-KLF6 (1: 3000; clone 2c11), mouse monoclonal anti-α-tubulin (Sigma−Aldrich; 1:2000), mouse anti β-actin (Sigma−Aldrich; 1:5000) and anti-HIF-1α (BD Biosciences; 1:250). After washing, the blots were incubated with horseradish peroxidase-conjugated donkey anti-rabbit or sheep anti-mouse IgG secondary antibodies (Amersham Bioscience; 1:5000) in TBS-T, at room temperature for one hour. Protein-antibody complexes were visualized using an enhanced chemiluminescence detection system (SuperSignal West Pico; Pierce) and exposed to Kodak film.

2.6. Knockdown of HIF-1α in HTR8/SVneo cells

HIF-1α small-interfering RNA (siRNA) was introduced into HTR8/SVneo cells to down-regulate HIF-1α expression. Cells in suspension (1×10^5) were transfected with 40 nM of the specific HIF-1α siRNA (silencer predesigned siRNA, ID number 42840, Ambion Inc.), or the negative control siRNA (SCR, Silencer, Ambion Inc.) using the transfection reagent (siPORT NeoFX; Ambion Inc., Austin, TX). Cells were incubated in a standard incubator at 5% CO_2 and 20% O_2. Twenty-four hours after, culture medium was replaced with fresh RPMI 1640 medium (Invitrogen) supplemented with 10% v/v FBS and antibiotics, and cells were further incubated for one hour in either 20% or 0.2% oxygen.

2.7. Statistical analysis

Results are presented as mean ± SEM of three independent experiments performed in duplicates or triplicates. For multiple comparisons, one-way analysis of variance (ANOVA) was performed with the Dunnett's *post hoc* test. Statistical analyses were performed using GraphPad Prism 5.0 software and p-values <0.05 were considered statistically significant.

3. Results

3.1. KLF6 is expressed in villous and extravillous trophoblasts from placentas of uncomplicated and preeclamptic pregnancies

Immunohistochemistry assays were performed to localize KLF6 in sections of third trimester placentas from uncomplicated and preeclamptic pregnancies. The trophoblastic identity of the cells was confirmed by cytokeratin (CK) immunostaining in serial sections. Although this antigen is expressed by all cells of epithelial origin, the absence of maternal luminal/glandular epithelium during the final stages of gestation makes CK reactivity a suitable marker for locating extravillous trophoblasts among the other decidual cells which are CK-negative [24]. In the placentas from both uncomplicated and preeclamptic pregnancies, cells exhibiting morphological characteristics similar to CK-reactive villous and extravillous cytotrophoblasts showed positive reaction for KLF6 (Figs. 1 and 2). In addition, KLF6 immunoreactivity was also seen in stromal, endothelial, and muscle cells in the decidua, particularly in placentas from preeclamptic pregnancies (Fig. 2). Controls did not show any reactivity (Figs. 1E and 2G). Quantitative analysis of KLF6 positive cells showed higher immunointensity in preeclamptic (29.9 ± 2.8) than in healthy term (20.6 ± 7.3, p = 0.0008) placental bed samples.

3.2. Low oxygen tension increases KLF6 protein levels in human HTR8/SVneo cells and placental explants

We next evaluated whether KLF6 expression is regulated by oxygen in trophoblast cells. Western blot experiments revealed that KLF6 was present in control cultures of HTR-8/SVneo cells maintained in 20% oxygen, its expression was significantly increased (2.5 ± 0.4, $p < 0.05$) after one hour of hypoxia exposure (0.2% oxygen), and returned to near basal levels after longer periods (three and twenty hours). HIF-1α levels were markedly increased with the first hour of hypoxia, and remained high under low oxygen tension (Fig. 3). KLF6 levels were also induced in HTR8/SVneo cells exposed to 1% oxygen for one hour (data not shown). Thus, KLF6 expression is early and temporarily augmented by hypoxia in HTR8/SVneo cells.

In order to determine whether KLF6 protein levels are also regulated by oxygen in placental villi, normal term placental explants were cultured for twenty-four hours in an atmosphere of 20% oxygen and then transferred to 1% oxygen for one hour or maintained in 20% oxygen. A significant increase in KLF6 protein level was detected in tissues exposed to hypoxia compared to control conditions and, as expected, HIF-1α protein levels were also higher in explants exposed to 1% oxygen versus 20% oxygen (Fig. 4).

3.3. Reoxygenation modulates hypoxia-induced KLF6 expression in HTR8/SVneo cells

To further study the impact of oxygen on KLF6 expression, HTR8/SVneo cells were exposed to hypoxia for one hour and then reoxygenated for one or two hours, while control cultures were maintained for three hours in 20% oxygen. In these conditions, KLF6 and HIF-1α levels showed a significant increase after the first hour of hypoxia and returned to basal levels after one or two hours of reoxygenation (Fig. 5). Accumulation of HIF-1α in hypoxia, followed by its disappearance upon reoxygenation, confirmed the experimental conditions.

3.4. HIF-1α is required for the hypoxia-mediated increase in KLF6 expression

Since HIF-1α plays an essential role in cellular responses to hypoxia we evaluated whether it was involved in the hypoxia-induced increase in KLF6 expression. To this end, cells were treated with a specific HIF-1α siRNA (siHIF) or a scrambled siRNA (SCR) as control. Twenty-four hours after transfection, cells were exposed to hypoxia or maintained in control conditions for one hour. In SCR-transfected cells, hypoxia induced KLF6 and HIF-1α expression (Fig. 6), in agreement with the results obtained using non-transfected cells (Fig. 3). As expected, treatment with siHIF prevented HIF-1α accumulation during exposure to hypoxia.

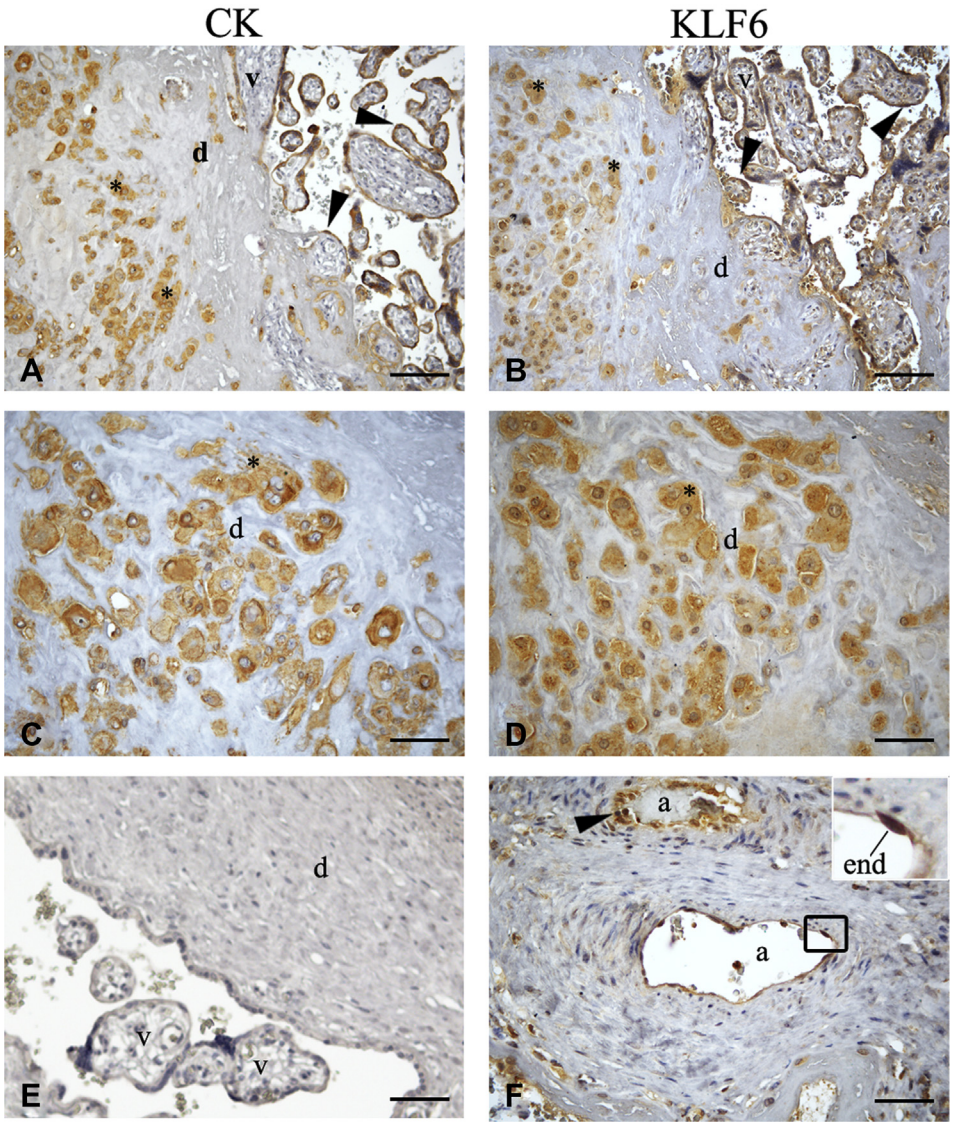

Fig. 1. Immunolocalization of CK (A, C) and KLF6 (B, D, F) in normal placental bed. (A, C) There was strong CK positivity in villous (v) trophoblast (syncytiotrophoblast, arrowheads) and in extravillous trophoblast cells (*) in decidua (d). **(B, D)** Arrowheads and asterisks highlight strong KLF6 immunoreactivity in similar CK+ structures (syncytiotrophoblast in villi and extravillous trophoblast cells in the decidua). **(F)** KLF6 immunolocalization is also seen in endothelial cells (end) from decidual vessels (a, artery). The region marked by the square is shown at higher magnification in the inset, where it is highlighted an endothelial KLF6 reactive cell. **(E)** Negative control is shown in human placental villi and decidua. Immunoperoxidase, Mayer's hematoxylin counterstaining. In A–B, E–F, scale bar = 50 μm; in C–D, scale bar = 25 μm.

Furthermore, exposure to hypoxia did not result in increased KLF6 expression in cells treated with siHIF (Fig. 6). Under well-oxygenated conditions (20% oxygen) HIF-1α silencing did not alter KLF6 basal expression compared to SCR-treated cells.

4. Discussion

Herein, we demonstrate that KLF6 is not only expressed in villous trophoblasts as already reported [21,25], but also in extravillous trophoblasts of placentas from uncomplicated and preeclamptic pregnancies. In addition, KLF6 is synthesized in stromal, endothelial, and muscle cells in the decidua and a higher KLF6 signal was detected in the placental bed of the preeclamptic group. KLF6 is also expressed in HTR8/SVneo extravillous cytotrophoblast cells and low oxygen tension induces an early and transient increase in KLF6 protein concentration. Moreover, hypoxia induces KLF6 expression in a HIF-1α dependent manner since HIF-1α silencing impairs KLF6 increase. However, basal KLF6 levels are detected in trophoblast cells in normoxia and even in HIF-1α-knockdown cells. Considering that oxygen tension plays an important role in trophoblast physiology and pathology [1–4] and that KLF6 regulates a pool of genes involved in proliferation, motility, invasion, angiogenesis, tissue repair and tissue response to stress under different scenarios [26–30], the present findings support the notion that KLF6 might contribute to placental development and anchoring to the uterus.

KLF6 expression was previously reported in villi and placental membranes from first, second, and third trimester human pregnancies [21,25]. In addition, herein we show expression in term extravillous cytotrophoblast and in HTR8/SVneo cells, a human first-trimester extravillous trophoblast cell line. In mouse KLF6 orthologous mRNA, mCPBP, was detected in the primitive placenta during all early placentation, whereas from 12.5 to 18.5 days postcoitum, mCPBP was strongly expressed in the spongiotrophoblast and labyrinth of the definitive placenta [31]. These data and our present results indicate that KLF6 is expressed in first trimester

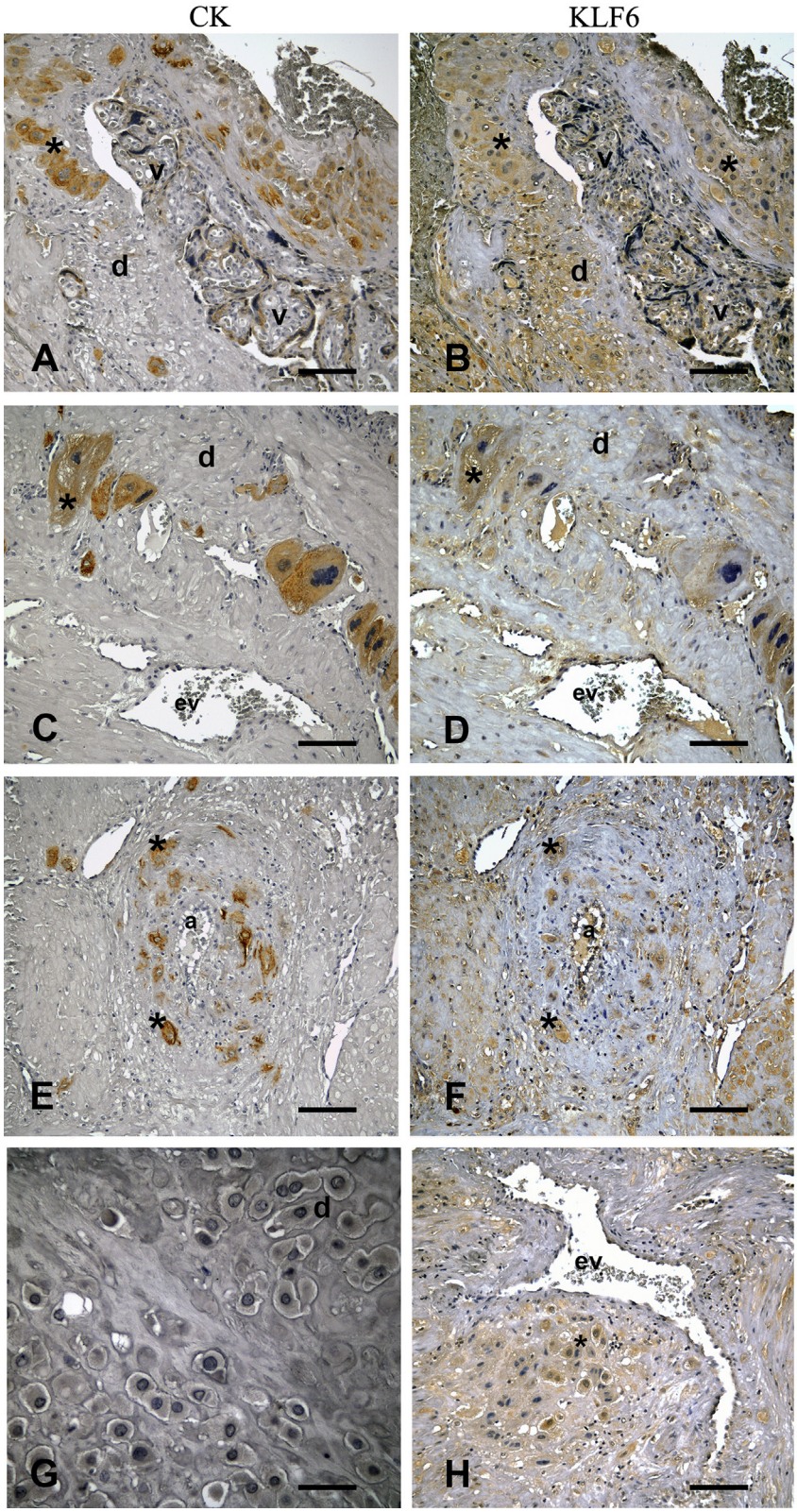

Fig. 2. Immunolocalization of CK (A, C, E) and KLF6 (B, D, F, H) in preeclamptic placental bed. Paraffin serial sections show reactivity for KLF6 **(B, D, F)** in cells also stained for CK **(A, C, E)** in villous (v) and in extravillous trophoblast cells (*) spread throughout the decidua (d) or located surrounding maternal vessels (a). Note, however, that KLF6 is also expressed by other cell types **(B, D, F, H)**. Negative control is shown in decidual tissue **(G)**. Immunoperoxidase, Mayer's hematoxylin counterstaining. In A–B, E–F, H, scale bar = 50 μm; in C–D, G, scale bar = 25 μm.

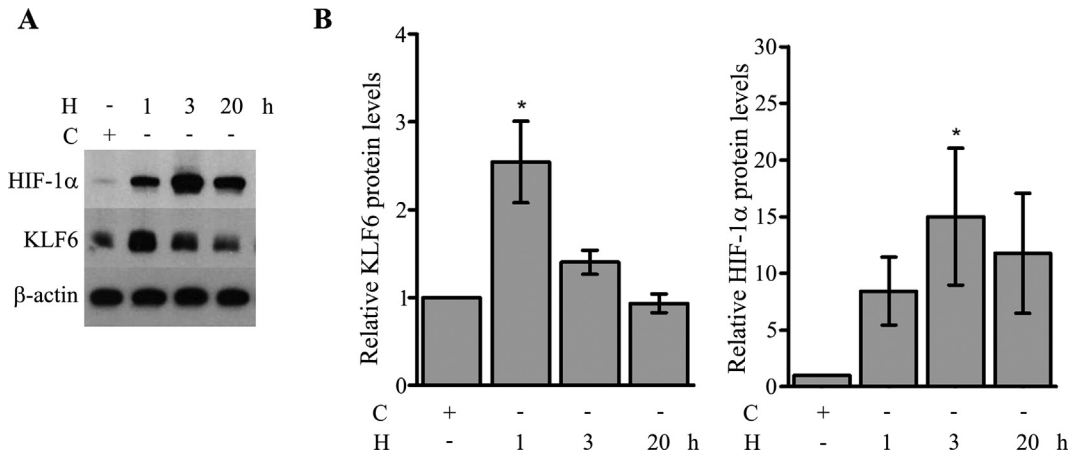

Fig. 3. KLF6 and HIF-1α expression in HTR8/SVneo cells exposed to hypoxia. Cells were exposed to hypoxia (H) for the time periods indicated or maintained in standard culture conditions for 20 h (C). Protein was extracted and Western blot analysis was performed for KLF6, HIF-1α, and β-actin (as loading control). **(A)** A representative blot of three independent experiments. **(B)** Relative protein levels of KLF6 and HIF-1α determined by densitometric analysis of the three independent experiments. Bars represent the mean ± SEM, (*p < 0.05 H vs C).

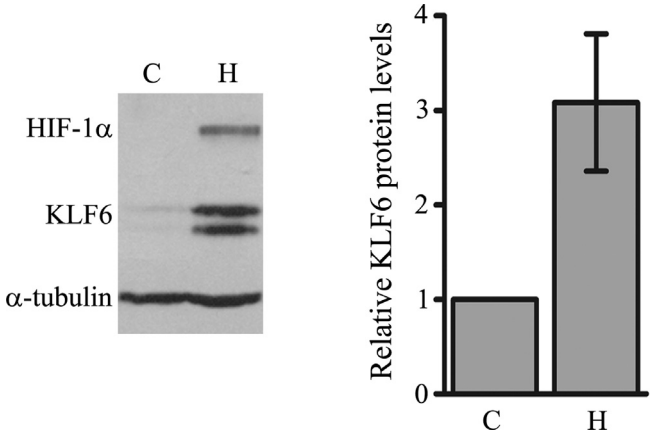

Fig. 4. Western blot analysis of KLF6 and HIF-1α expression in normal term placenta villi. Protein extracts from term placenta villi exposed to 1% oxygen (H) for 1 h or maintained in 20% oxygen (C) were subjected to Western blot analysis to evaluate KLF6 and HIF-1α protein levels relative to α-tubulin expression. The expression of HIF-1α confirmed the effect of hypoxia. One representative blot of triplicate assays performed with explants from two different placentas and its densitometric quantification is shown.

as well as term villous and extravillous trophoblasts.

Successful human placental development and pregnancy progression depend on adequate cell proliferation, migration, invasion, and remodelling of maternal uterine structures by extravillous trophoblast cells. Numerous factors have been reported to actively control these processes including environmental factors such as oxygen tension [4,32]. Remarkably, many of them are known transcriptional targets of KLF6 in other cell contexts. Among them are E-cadherin, urokinase plasminogen activator, endoglin, TGF-β1, TGF-β receptor type I, VEGF, and metalloproteinase 9 (MMP9) [15,33,34]. MMPs are associated with cell invasion and tissue remodeling in a variety of biological systems, including extravillous trophoblast cells [35]. Interestingly, it has been recently reported that the MMP14 gene is directly upregulated by KLF6 after vascular injury resulting in the release of soluble endoglin [36]. Remarkably, soluble endoglin is intimately associated with systemic hypertension during pregnancy, contributing to the pathogenesis of preeclampsia [37]. In addition, hypoxia, HIF-1α, and dysregulation of some of the above mentioned genes are associated with reduced trophoblast invasion, spiral arteries remodeling, and an imbalance in angiogenic and anti-angiogenic factors characteristic of preeclampsia [38]. Thus, it is possible that placental KLF6 contributes to placental tissue and vascular remodelling in normal as well as preeclamptic pregnancies.

Much has been discussed regarding the *in vitro* oxygen concentration that most accurately mimics the *in vivo* situation. Although no *in vitro* system completely replicates the *in vivo* situation, our study demonstrates that KLF6 expression responds to changes in oxygen levels in immortalized first-trimester trophoblast HTR8/SVneo cells and in term placental explants. It is generally accepted that 20% oxygen represents a hyperoxic condition for the placenta, thus KLF6 protein levels observed in 20% oxygen explants may not fully represent KLF6 level in placental normoxia. Nevertheless, the results observed in the placental explant model further suggest that KLF6 expression may be regulated by oxygen in the placenta *in vivo*.

Our results are in line with those recently reported by Koizume et al. who described that *KLF6* is up-regulated by exposure to 1% oxygen or $CoCl_2$-induced hypoxia in a HIF-1α- and Sp1-dependent manner in the OVSAYO ovarian clear cell carcinoma cell line. These authors also demonstrate, that KLF6 overexpression leads to transactivation of the intercellular adhesion molecule-1 (ICAM1) [39]. Although the role of ICAM1 in the pathophysiology of preeclampsia has not been fully elucidated, significantly increased levels of this molecule have been found in placentas from preeclamptic pregnancies compared to controls [40]. Considerable evidence supports an important role of HIF signalling in placental development, hypoxia signaling, and the regulation of downstream mediators of preeclampsia [3,6,38,41]. To date, there are more than 100 HIF-1 downstream genes identified with varying functions [42]. Interestingly, endothelin-1, a direct target of HIF-1, is a known activator of KLF6 expression [43], indicating that KLF6 could be not only a direct downstream effector of HIF-1 but also an indirect target. Further studies should address whether a HIF-1α/KLF6 pathway regulates the expression of genes involved in trophoblast invasion and/or angiogenesis in normal placental development and in the pathophysiology of preeclampsia.

Likewise to the increase in KLF6 expression during villous trophoblast differentiation [21], herein we observed a rapid and transient increase in KLF6 protein levels following hypoxia

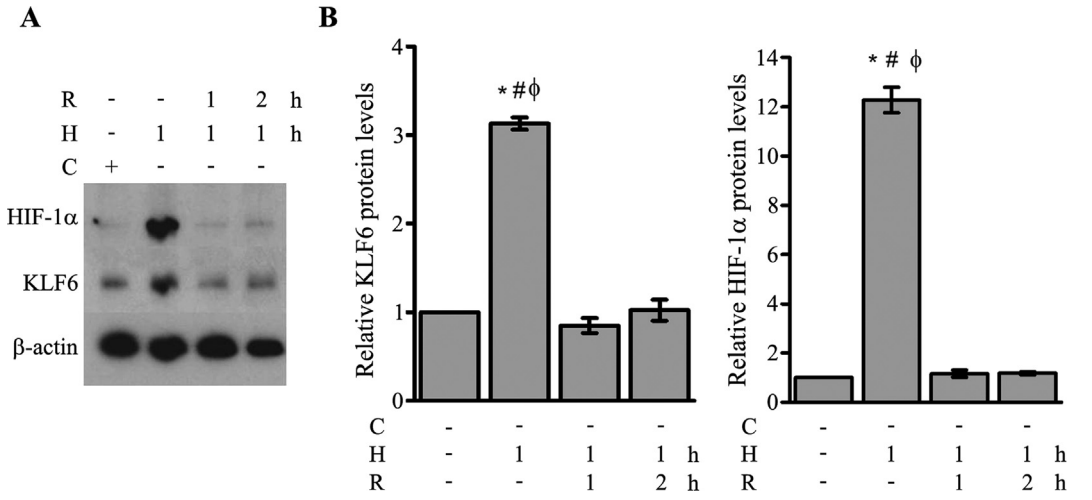

Fig. 5. KLF6 and HIF-1α expression in HTR8/SVneo cells exposed to hypoxia/reoxygenation. Cells were exposed to hypoxia (H) for one hour, or to hypoxia (one hour) followed by reoxygenation (R) for the indicated time periods, or maintained in 20% O_2 for three hours (C). **(A)** A representative Western blot for KLF6, HIF-1α, and β-actin (loading control) of three independent experiments. **(B)** Relative protein levels of KLF6 and HIF-1α estimated by densitometric analysis of the three independent experiments. Data are expressed as mean ± SEM (*, #, ϕ $p < 0.05$ vs C, H/R 1 h, H/R 2 h, respectively).

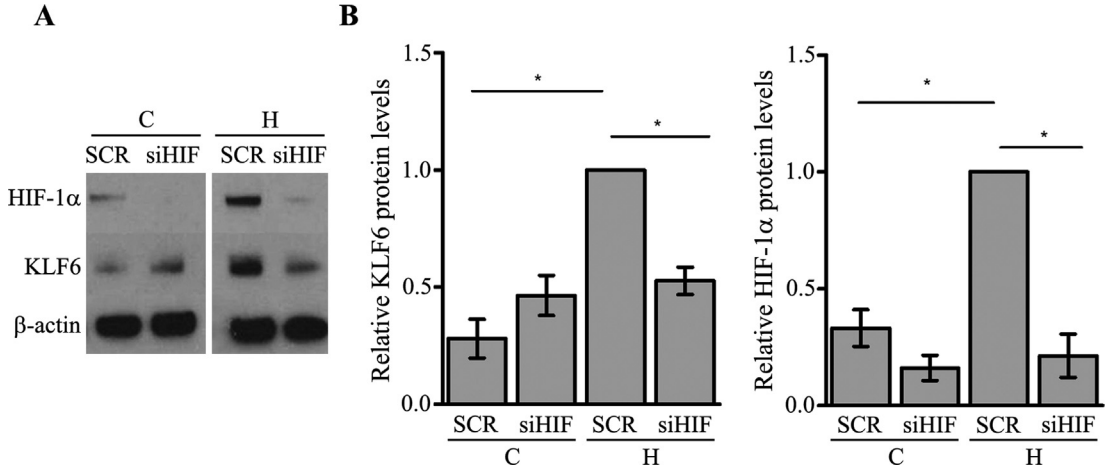

Fig. 6. KLF6 expression in HIF-1α silenced cells. HTR8/SVneo cells were transfected with a specific HIF-1α siRNA (siHIF) or a negative control siRNA (SCR). Twenty-four hours later, cells were exposed to hypoxia for one hour (H) or maintained in 20% O_2 (C) and then protein extracts were subjected to Western blot analysis. **(A)** A representative immunoblot for KLF6, HIF-1α, and β-actin (loading control) of three independent experiments. **(B)** Relative protein levels of KLF6 and HIF-1α estimated by densitometric analysis of the three independent experiments, expressed as fold change relative to levels detected in cells transfected with SCR and exposed to hypoxia. Data are expressed as mean ± SEM (*$p < 0.05$).

exposure. Interestingly, KLF6 is rapidly induced after liver injury in stellate cells, after renal ischemia/reperfusion in the mouse kidney, and following treatment with serum or phorbol ester in several cell lines [14,29,44]. Moreover, it is regulated by endothelin-1 and H_2O_2 as an immediate early response gene in neonatal rat cardiac myocytes [43]. Thus, *KLF6* might be an important early response gene that fine tunes the response not only to hypoxia but to other stress signals in order to allow cell survival and homeostasis.

In summary, our data demonstrate for the first time that KLF6 is expressed in extravillous trophoblasts, and that its protein level is regulated by oxygen in a HIF-1α dependent manner.

Conflict of interest statement

The authors Racca AC, Ridano ME, Bandeira CL, Bevilacqua E, Avvad Portari E, Genti-Raimondi S, Graham CH, and Panzetta-Dutari GM declare that no conflict of interest exists for the manuscript submitted.

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