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

A.C. Racca [a], M.E. Ridano [a], C.L. Bandeira [b], E. Bevilacqua [b], E. Avvad Portari [c], S. Genti-Raimondi [a], C.H. Graham [d, 1], G.M. Panzetta-Dutari [a, *, 1]

[a] Centro de Investigaciones en Bioquímica Clínica e Inmunología (CIBICI-CONICET), Departamento de Bioquímica Clínica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Córdoba, Argentina
[b] Department of Cell and Developmental Biology, Institute of Biomedical Sciences, University of São Paulo, Brazil
[c] Department of Pathology at Medical Sciences School, State University of Rio de Janeiro, Brazil
[d] Departments of Biomedical and Molecular Sciences and Urology, Queen's University, Kingston, Ontario, Canada

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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.

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].

* Corresponding author. CIBICI-CONICET, Departamento de Bioquímica Clínica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Haya de la Torre y Medina Allende, Ciudad Universitaria, X5000HUA, Córdoba, Argentina.
E-mail address: gpan@fcq.unc.edu.ar (G.M. Panzetta-Dutari).
[1] Equal contribution.

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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.