Hypoxia-inducible factor 2 alpha impairs human cytotrophoblast syncytialization: new insights in placental dysfunction and fetal growth restriction

Running title: HIF-2α in placental dysfunction

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NONSTANDARD ABBREVIATIONS LIST

ARNT	Aryl hydrocarbon receptor nuclear translocator
FGR	Fetal Growth Restriction
GCM1	Glial cells missing transcription factor 1
hCG	Human chorionic gonadotrophin
HDAC	Histone deacetylase
HIF	Hypoxia-inducible factor
PE	Preeclampsia
PGF	Placental growth factor
PHD	Prolyl-4-hydroxylase
pVHL	von Hippel-Lindau protein
siRNA	Small interfering RNA
SDHA	Succinate dehydrogenase A
sFlt-1	Soluble fms-like tyrosine kinase-1
TBP	TATA-box binding protein

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CONFLICT OF INTEREST STATEMENT

The authors report no conflict of interest.

AUTHOR CONTRIBUTIONS

A.C. and C.D. designed and performed research; F.D. and P.S. designed research and analyzed data. A.C. and P.B. described clinical samples. All authors were involved in writing the paper and had final approval of the submitted and published versions.

ABSTRACT

Insufficient remodeling of uterine arteries causes pregnancy-related diseases, including fetal growth restriction and preeclampsia. In these situations, reduced maternal blood flow in the placenta is thought to be responsible for the persistence of a low oxygen environment throughout pregnancy. We hypothesized that a chronic activation of transcription factors hypoxia-inducible factors (HIFs) actively participate in placental underdevelopment, which impairs fetal growth. The computer-assisted analysis in pathological placentas revealed an increased number of HIF-2α-positive nuclei in the syncytium compared to normal human placentas, while HIF-1α stabilization was unchanged. Specific involvement of HIF-2α was confirmed in primary human cytotrophoblasts rendered deficient for *HIF1A* or *HIF2A*. Silencing *HIF2A* increased the expression of main syncytialization markers as well as differentiation and syncytium formation. It also improved placental growth factor bioavailability. None of these changes was seen when silencing *HIF1A*. Conversely, the experimental induction of HIF-2α expression repressed forskolin-induced differentiation in BeWo choriocarcinoma cells. Our mechanistic insights evidence that transcription factor HIF-2α impairs placental function, thus suggesting its participation in fetal growth restriction when placentas become chronically hypoxic. Furthermore, it suggests the possibility to develop novel molecular targeting therapies for placental dysfunction.

KEYWORDS

placenta, trophoblast, hypoxia, Fetal Growth Retardation, preeclampsia

INTRODUCTION

Fetal Growth Restriction (FGR) is one of the most common pregnancy complications, affecting around 9% of all pregnancies.[1] It is one of the main challenges for obstetricians and pediatricians, as low-birthweight infants have a higher risk of stillbirth and perinatal death as well as morbidity, including worse neurodevelopmental outcomes, respiratory distress syndrome and infections.[2-4] By adulthood, growth restricted infants are more prone to develop hypertension, coronary artery disease, diabetes, metabolic syndrome, and dyslipidemia, emphasizing FGR as a huge issue in our health care systems.[5,6] Unfortunately, few therapeutic options are currently available and preterm delivery remains the major strategy in severe cases.[7]

FGR can have many causes, but the majority are thought to arise from placental insufficiency, which may, in turn, be linked to the development of preeclampsia (PE), a major hypertensive disorder of pregnancy.[8] The kinetics of placental and fetal growth are closely correlated, as fetal growth is related to nutrient supply, itself dependent on placental development and functions.[9] These functions are fulfilled by the syncytium, a multinuclear layer, which constitutes the interface between the maternal and fetal circulation. Throughout pregnancy, the syncytium is formed by the fusion of mononuclear villous cytotrophoblasts to form plurinuclear syncytiotrophoblasts, a process called syncytialization.[10]

Early placental development occurs under a low oxygen environment (~2.5%) that regulates the invasion of the uterine wall by the extravillous trophoblasts.[11,12] After 12 weeks of pregnancy, the maternal blood enters the intervillous space and the placental pO_2 progressively reaches a similar level than the arterial pO_2 (~8%).[13,14] The remodeling of the uterine arteries by the extravillous trophoblasts reduces vessel pulsatility, allowing to obtain a steady blood flow that ensures perfusion of the intervillous space and adequate transit time for exchange.[15] Even if a low-oxygen environment during the 1st trimester is critical for regulating placentation, it is currently recognized that insufficient remodeling of the uterine arteries causes placental insufficiency, leading to FGR and PE.[16,17] In these cases, reduced or intermittent blood flow in the placenta is thought to be responsible for persistent hypoxia and oxidative stress during the second half of gestation.[18]

In mammalian cells, the main regulators of gene expression under hypoxia are hypoxia-inducible factors (HIFs).[19] They act as heterodimers constituted by a variable α subunit and a common β subunit (ARNT). While the β subunit is constitutively expressed, α subunits are transcribed but subject to posttranslational stability regulation. Under normoxia, prolyl-4-hydroxylases (PHD) using molecular oxygen as a co-substrate indeed hydroxylate α subunits at conserved proline residues.[20] Once prolines are hydroxylated, the von Hippel-Lindau protein (pVHL) complex ubiquitylates these α subunits, addressing them to proteasomal degradation.[21,22] In mammals, three different genes encode HIF-α subunits. While HIF-1α

expression is ubiquitous, the expression of its paralogs HIF-2α and HIF-3α is more restricted, suggesting that they could play more specialized roles.[23]

HIF-1α and HIF-2α are highly expressed in the human placenta. Their abundance during the first trimester of pregnancy is consistent with the low oxygen environment of the placenta.[24] Interestingly, a higher expression of HIF-1α was found in PE and FGR placentas compared to normal term pregnancies, as well as a direct inverse association between circulating HIF-1α and PGF in serum of PE patients.[25,26] However, their roles in placental development and trophoblast differentiation during the second and the third trimesters is not completely elucidated.

Given the fact that oxygen tension normally rises after 10-12 weeks of gestation and that the fetus mainly grows during this period, our hypothesis is that persistent HIF activation by persisting low oxygen environment in the placenta related to abnormal placentation would impair syncytial formation, hence inducing placental dysfunction and compromising fetal growth. In this study, we aimed to unravel the roles of HIF-1α and HIF-2α in impaired syncytialization and placental development by using human placentas and human primary cytotrophoblasts.

MATERIAL & METHODS

Placenta collection

We first conducted a retrospective monocentric study on placentas collected from normal and pathological pregnancies. This study was conducted with the approval of the Ethical Committee of the Université catholique de Louvain, Brussels (ref. 2018/23OCT/397). Births in Cliniques universitaires Saint-Luc (Brussels, Belgium) from 2018 were included (**Figure S1**). Gestational age was estimated from the first day of the last menstrual period and confirmed by first trimester ultrasound examination. The diagnosis of FGR was defined by a birth weight according to sex and gestational age less than the 10th percentile of the standard growth curve for the European population.[27] Pregnancies associated to factors that could have influenced fetal growth were excluded except PE, that was considered as a second group of patients. The control group was constituted by uncomplicated pregnancies delivering neonates with birth weights within the 10th and 90th percentile. Clinical characteristics of the subjects are shown in **Table 1**. Histologic characterization of the samples was independently realized by two experienced pathologists following the Amsterdam Placental Workshop Group Consensus Statement.[28]

Tissue staining quantification

For quantitative evaluation in whole tissue sections, histologic slides from recruited placentas were incubated with primary antibodies (**Table S1**) overnight at 4°C. Immunohistochemical reactions were visualized with the horseradish peroxidase technique (DAB, Dako, Agilent Technologies). Colorectal tumor tissue sections were used as positive controls, while negative controls consisted in slides incubated with secondary antibodies alone. Stained slides were digitalized using a SCN400 slide scanner (Leica Biosystems, Wetzlar, Germany) at X20 magnification. Scanned slides were analyzed using the image analysis tool author version 2017.2 (Visiopharm, Hoersholm, Denmark) following the workflow described in **Figure S2**.

Primary cytotrophoblast isolation

For primary cell culture, 5 normal placentas were collected from uncomplicated pregnancies immediately after elective cesarean section at term. Primary cytotrophoblasts were isolated following a previously validated protocol.[29] Briefly, the placenta was cut into 1 cm^2 pieces and digested at 37 °C with dispase II and DNase I, grade II (Roche, Mannheim, Germany). After sequential filtration, the cells were separated by density Percoll gradient centrifugation (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The cells were then counted with a hemocytometer and the viability was assessed by Trypan Blue exclusion (Sigma-Aldrich,

Steinheim, Germany). The average yield was 208 × 106 (± 59 × 106) cytotrophoblasts for 72 g of tissue treated.

Cell culture and forskolin treatment

Primary cytotrophoblasts were cultured in Iscove's Modified Dulbecco's Medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) complemented with 50 µg/mL gentamicin (Carl Roth GmbH & Co., Karlsruhe, Germany) and 10% FBS (Gibco, Thermo Fisher Scientific). Cultures were maintained for 5 days in two separate atmosphere-controlled humidified incubators, either under 21% O_2/5% CO_2 or 2.5% O_2/5% CO_2, at 37°C balanced with N_2. Human choriocarcinoma cells 98 (BeWo) ATCC® CCL-98™ were cultured in 10% FBS-enriched Ham's F-12 medium (Sigma-Aldrich, Saint-Louis, MO, USA), and maintained at 37°C under humidified conditions of 21% O_2/5% CO_2. Forskolin (Fisher Scientific, Thermo Fisher Scientific) was used to induce syncytialization at a concentration of 25 µM for 24 h.

siRNA-mediated HIF-α silencing

Eighteen hours after isolation, primary cytotrophoblasts were transfected with 50 nM of cocktails of small interfering RNA (siRNAs) specifically targeting either *HIF1A* or *HIF2A* (sc-35561 and sc-35316, respectively; Santa Cruz Biotechnology, Dallas, TX, USA) using the Lipofectamine RNAiMAX Transfection Reagent (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's protocol.

Generation of stabilized mutated HIF-2α

A plasmid encoding a stabilized version of HIF-2α was generated by site-directed mutagenesis using the QuickChange Lightning site-directed kit (Agilent Technologies, Santa Clara, CA, USA). Two pairs of mutagenic primers (**Table S2**) were designed in order to replace prolines 405 and 531 by alanines in the oxygen-dependent degradation domain of *HIF2A*, using the commercially available human *HIF2A* expression vector pFN21A[HIF-2α] as a template (Promega, Madison, WI, USA). The ability of the mutated protein to activate the Hypoxia Response Element (HRE) was assessed by a dual-luciferase reporter assay 24 h after transfection (Promega). To do so, BeWo cells were co-transfected with pGL4.42[luc2P-HRE] (ref. E4001, Promega) and mutated pFN21A[HIF-2α] vectors using Lipofectamine 3000 Reagent (Invitrogen, Thermo Fisher Scientific).

Gene expression

Total RNA was extracted from cells using Roti-Prep RNA MINI (Carl Roth GmbH & Co.), followed by reverse transcription using qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MA, USA). Quantitative real-time PCR (RT-qPCR) was performed with 5 ng of

cDNA and 0.2 µM of specific primers (**Table S3**) in Takyon ROX SYBR 1X MasterMix dTTP blue (Eurogentec, Seraing, Belgium) on a StepOne Real-time PCR System (Applied Biosystems, Thermo Fisher Scientific). Relative gene expression was calculated with the Pfaffl method, using succinate dehydrogenase A (*SDHA*) and TATA-box binding protein (*TBP*) as reference genes [30,31].

Western blotting

Whole cell lysates and nuclear protein extracts were prepared using M-PER Mammalian Protein Extraction Reagent and NE-PER Nuclear and Cytoplasmic Extraction Reagents kits, respectively (Thermo Fisher Scientific). Twenty micrograms of proteins were reduced and loaded onto a 10% Acrylamide/Bisacrylamide Bolt Bis–Tris mini gel (Invitrogen, Thermo Fisher Scientific) and separated for 22 min at 200 V. Proteins were transferred onto an Invitrolon PVDF membrane (Invitrogen) for one hour at 20 V. Then, the membrane was blocked for one hour in 5% non-fat dry milk in 1X TBS-T (50 mM Tris/Tris–HCl, 138 mM NaCl, 20 mM KCl, and 0.1% Tween-20). The membrane was incubated overnight at 4 °C with the primary antibodies and then for one hour at room temperature with the HRP-conjugated secondary antibodies (**Table S1**). Bound antibodies were detected using the SuperSignalTM West Pico PLUS chemiluminescent substrate (Thermo Fisher Scientific), and Amersham HyperfilmTM ECL films (GE Healthcare Limited).

Secreted protein quantification

Supernatants of the last 24 h of culture were collected to quantify secreted placental growth factor (PGF) with a human PGF Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA), human soluble fms-like tyrosine kinase-1 (sFlt-1) with a VEGFR1/Flt-1 Quantikine ELISA Kit (R&D Systems), and β human chorionic gonadotropin (βhCG) with a B.R.A.H.M.S. Kryptor Compact Plus immune analyzer (Thermo Fisher Scientific). Whole cell lysates from the same well as the supernatant were prepared using M-PER (Thermo Fisher Scientific) in order to normalize secreted proteins to total proteins.

Fusion index

After 5 days of culture on CELLview slides (Greiner Bio One, Vilvoorde, Belgium), cytotrophoblasts were fixed and stained with antibodies listed in **Table S1**. Nuclei were counterstained with NucBlue when mounting slides with ProLong Glass Antifade Mountant (Invitrogen). Fluorescence was examined with an AxioImager microscope combined with ApoTome, using the Axiovision software (Zeiss, Oberkochen, Germany). The fusion index was calculated as (N-S)/T, where N was the total number of nuclei in syncytia (cells containing ≥3 nuclei), S the total number of syncytia and T the total number of nuclei.[32]

Statistics

All data show means ± SEM. *N* represents the number of individual experiments and *n* the total number of biological replicates. Results were normalized to the control condition under 21% O_2. Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA) using Student's t-test, Mann-Whitney test and one-way ANOVA followed by a Dunnett's multiple comparisons test when appropriate. $P < 0.05$ was considered to be statistically significant.

RESULTS

HIF-2α, but not HIF-1α, protein expression is induced in human pathological placentas from fetal growth restriction and preeclamptic patients

In a retrospective study, we first analyzed human pathological placentas from normal (control, *N* = 11), FGR (*N* = 12) and FGR + PE (PE; *N* = 8) patients (**Table 1**). Because fetal and placental growth are directly correlated, neonates and pathological placentas in FGR group showed a significantly reduced weight compared to control placenta from same gestational age (**Table 2**).[33] In our cohort, the different histologic signs of altered maternal vascular perfusion were more often encountered in placentas from FGR and PE groups, which is consistent with the fact that insufficient remodeling of maternal arteries is thought to be responsible for chronic hypoxia along pregnancy (**Table 2**).[8]

HIF-1α and HIF-2α expression was quantified in the syncytium of these placentas by using immunohistochemistry. Nuclear HIF-1α expression did not differ between control and pathological placentas (**Figure 1a**). Comparatively, the nuclear expression of HIF-2α was significantly higher in pathological placentas (16.64% ± 4.79% of HIF-2α-positive nuclei in the FGR group and 18.94% ± 5.11% in the PE group) compared to controls (5.02 ± 0.96%) (**Figure 1b**), which represented an increase by > 3 folds. Given the difference in median gestational age between the control and the PE group, the influence of this parameter on HIF-2α expression was questionable. However, we did not observe significant correlation between the gestational age and HIF-2α quantification inside the PE group, with no statistical difference between the two extreme gestational ages (26 *vs.* 37 weeks).

Given the fact that it is a qualitative study, it is difficult to draw correlations between HIF-2α quantification and any of the pathological features analyzed. Yet, syncytial knots and fibrin deposits seemed to be correlated with HIF-2α stabilization, as the placentas with a very low HIF-2α expression level did not show any of these two characteristics. Altogether, these observations indicated that HIF-2α could play a predominant role over HIF-1α in human pregnancy-related diseases.

Chronic hypoxia impairs cytotrophoblast syncytialization

To investigate the influence of the local pO_2 on syncytialization, we exposed primary human cytotrophoblasts from normal placentas at term to high (normoxia, 21%) or low (hypoxia, 2.5%) oxygen levels according to our previous publications.[34-36] As a marker of biochemical differentiation, hCG production was analyzed by RT-qPCR and ELISA. The *CGB* (βhCG subunit mRNA) expression increased after 5 days of culture under 21% O_2, but not under 2.5% O_2 (**Figure 2a**). As expected from this observation, the concentration of free βhCB was higher in the supernatant of normoxic cells (**Figure 2b**). Reduced biochemical differentiation was

linked to impaired morphological changes, as hypoxia decreased the fusion index of cytotrophoblasts by 12.44% (**Figure 2c-e**).

Inhibition of cytotrophoblast syncytialization under hypoxia is dependent on HIF-2α

To investigate the potential contributions of HIF-1α and HIF-2α to the hypoxia-dependent inhibition of primary cytotrophoblast differentiation, *HIF1A* and *HIF2A* were silenced by transfecting the cells with a cocktail of specific siRNAs. *HIF1A* and *HIF2A* mRNA expression was decreased by 55.93 ± 7.24% (**Figure S3a**) and 49.45 ± 12.88% (**Figure S3b**), respectively, which was confirmed at the protein level using western blotting (**Figure S3c,d**). When *HIF2A* was silenced under hypoxia, *CGB* expression and βhCB protein secretion were significantly increased (**Figure 3a, b**). Comparatively, *HIF1A* silencing did not significantly modify these parameters, indicating that HIF-2α, not HIF-1α, controls biochemical cytotrophoblast differentiation.

Based on these observations, we hypothesized that changes in biochemical differentiation were linked to changes in cell fusion. We therefore analyzed the expression of 4 specific markers of syncytialization: twist-related protein 1 (TWIST1), glial cells missing 1 (GCM1), syncytin-1 (*ERVW1*) and syncytin-2 (*ERVFRD1*). TWIST1 is a transcription factor that has been described to enhance the expression of GCM1, another transcription factor promoting cytotrophoblast syncytialization.[37,38] GCM1 is a key element of several signaling pathways leading to increased expression of syncytins.[39] Syncytin-1 and syncytin-2 are membrane-bound proteins essential for cell fusion.[40] In wild-type primary cytotrophoblasts, the mRNA expression of all 4 markers was decreased when the cells were exposed to chronic hypoxia *versus* normoxia (**Figure 3c-f**). Silencing *HIF1A* did not rescue their transcription. However, silencing *HIF2A* under hypoxia almost completely restored *TWIST1*, *GCM1*, *ERVW1* and *ERVFRD1* mRNA expression to normoxic levels. Morphological changes were assessed by immunofluorescence. Consistently, reducing HIF-2α expression under hypoxia increased the formation of the syncytium, as illustrated by the percentage of fused cells increasing from 20.01 ± 2.95% to 29.81 ± 2.78% ($P < 0.01$; **Figure 3g, h**). In the same conditions, reducing HIF-1α expression had no effect.

Oxygen-resistant, mutated HIF-2α impairs BeWo syncytialization

While our above data evidenced that *HIF2A* silencing under hypoxia restores normal cytotrophoblast differentiation and syncytialization, we decided to confirm this paradigm by the opposite approach consisting in overexpressing *HIF2A* in normoxic placental cells. Using site-directed mutagenesis, we produced a constitutively expressed version of human HIF-2α by replacing prolines 405 and 531 by alanines in the oxygen-dependent degradation domain of

HIF-2α (**Figure 4a**). Mutated HIF-2α was resistant to degradation under 21% O_2 and kept its ability to transactivate the Hypoxia Response Elements (HRE) of a reporter plasmid (**Figure S4a, b**).

To focus on the syncytialization process, we used BeWo human choriocarcinoma cells that, contrary to primary human cytotrophoblasts, do not readily fuse in culture. In this manner, we could transfect the cells with HIF expression vectors before inducing syncytialization with forskolin.[41] As expected, forskolin strongly increased *CGB* expression (**Figure 4b**) and βhCG secretion (**Figure 4c**) compared to the control treatment. In the same conditions, overexpressing the constitutively active version of human HIF-2α significantly inhibited syncytialization, as illustrated by decreases in *CGB* expression and free βhCG secretion. This set of data thus confirmed that HIF-2α activation *per se* is sufficient to restrict normal placental development.

Placental growth factor bioavailability is decreased by HIF-2α

Finally, we considered angiogenic factors produced by syncytiotrophoblasts, in particular the soluble fms-like tyrosine kinase-1 (sFlt-1):placental growth factor (PGF) ratio that is a well-known predictive marker of placenta-related disorders [42]. In wild-type primary human cytotrophoblasts, hypoxia decreased *PGF* but enhanced *FLT1* transcription (**Figure 5a,b**). It also reduced the secretion of the corresponding proteins (**Figure 5c,d**), resulting in a high sFlt-1:PGF ratio (**Figure 5e**). Silencing *HIF1A* had no effect on *PGF* mRNA expression (**Figure 5a**) and PGR protein secretion (**Figure 5c**); it significantly decreased *FLT1* mRNA expression (**Fig. 5b**) but not sFlt-1 protein secretion (**Figure 5d**), resulting in a similarly high sFlt-1:PGF ratio than in wild-type cells (**Figure 5e**). Comparatively, silencing *HIF2A* had profound effects on all parameters. It strongly increased *PGF* gene (**Figure 5a**) and PGF protein (**Figure 5c**) expression, while decreasing *FLT1* gene expression (**Figure 5b**) and sFlt-1 protein (**Figure 5d**) secretion, resulting in a ~6-fold reduction in the sFlt-1:PGF ratio (**Figure 5e**). This finding highlights the potential involvement of HIF-2α in the pathophysiology of endothelial dysfunction associated to FGR and PE.

DISCUSSION

This study evidences that HIF-2α, but not HIF-1α, plays a major role in human cytotrophoblast syncytialization. In clinical samples, HIF-2α is stabilized in the placentas of FGR and preeclamptic patients compared to normal placentas. Conversely, using silencing and overexpression approaches, we demonstrated that HIF-2α represses biochemical cytotrophoblast differentiation and fusion, as well as the sFlt-1:PGF ratio, a predictive marker of placenta-related disorders.[42] Overall, our study suggests that HIF-2α could be a target for future therapies aimed at countering FGR and PE.

During the first trimester of pregnancy, the oxygen environment of the placenta is crucial for the remodeling of maternal arteries by invasive extravillous trophoblasts. Indeed, using human first-trimester trophoblasts, Wakeland *et al.* recently showed that hypoxia promotes extravillous trophoblast proliferation and inhibits syncytiotrophoblast differentiation in a HIF-dependent manner.[12] However, the situation is slightly different when studying the placenta at term. As confirmed by our findings, the culture of term cytotrophoblasts under less than 5% O_2 led to a reduction in *GCM1* expression, along with a reduction of cell-cell fusion.[43,44]

In humans, Sheridan et al. reported that histological sections of PE placentas showed a higher number of cytotrophoblasts and a lower syncytiotrophoblasts/cytotrophoblasts ratio than normal placentas, suggesting that syncytialization was impaired in these samples.[45] Similarly to PE placentas, histologic analyses of FGR placentas suggested placental underdevelopment, including villous hypermaturity and distal villous hypoplasia.[46] Interestingly, primary cytotrophoblasts isolated from placentas of PE and FGR pregnancies showed impaired syncytialization compared to normal pregnancies.[47,48] Analysis of FGR and PE placentas showed reduced GCM1, syncytin-1 and syncytin-2 expression and defective syncytialization *ex vivo*.[49-52] In particular, Deyssenroth *et al.* showed that *GCM1* was among the genes with the largest negative effect size on fetal growth when screening the expression of placental regulatory genes associated with FGR by custom-designed microarray.[53] Interestingly, this study also showed that *HIF2A* was associated with increased odds of FGR status. Immunostaining of pathological placentas have also confirmed that HIF-2α is stabilized in FGR or preeclamptic pregnancies, but computer-assisted quantification has not been realized.[54,55] Our study fills this gap, as it quantitatively confirms these data: HIF-2α is indeed overexpressed in the nuclei of pathological syncytiotrophoblasts. We further report that increased HIF-2α activity (*per se* or as a result of hypoxia) is sufficient to induce many molecular signs typical of these pathologies. *In vivo*, Albers *et al.* provided interesting cause and effect evidence between placental dysfunction and HIF-1α by showing that constitutively active HIF-1α expression, specifically in mouse trophoblasts, results in PE-like symptoms and FGR.[56] However, as HIF-1α and HIF-2α are able to transactivate similar DNA core sequence, and in the absence of similar experiment conducted with HIF-2α expression, these results only support the role playing by HIFs in placental dysfunction.

The molecular signaling pathway(s) by which HIF-2α represses syncytialization remain(s) to be elucidated. The existence of different isoforms of HIF-α subunits raises the question about the specificity of their action as well as the spectrum of target genes regulated by both subunits. Even if HIF-2α shares 48% sequence identity with HIF-1α and binds to a common core sequence, both subunits differ in their transactivation domains, implying they have an inherent property that determines its binding distribution across the genome as well as distinct

transcriptional cofactors, possibly expressed in a tissue-specific manner.[57-59] In addition, HIF-2α is hydroxylated at a much lower efficiency than HIF-1α, which results in its preferred stabilization at higher oxygen tensions and during prolonged hypoxic exposure.[60] Transcriptomic studies already identified differentially upregulated genes by HIF-1α and/or HIF-2α in several cell types, but to date, similar experiments were not performed in human cytotrophoblasts.[61-63] Complementarily to target gene modulation, HIFs are also known to induce epigenetic modifications. For example, it has been shown in trophoblast stem cells that histone deacetylase (HDAC) activity is increased by HIFs and determines stem cell fate in the placenta.[64] Complementarily, Chang *et al.* demonstrated that HDAC5 facilitates GCM1 deacetylation and suppresses its transcriptional activity, thus inhibiting syncytialization.[65] Regarding these results, a deeper understanding of specific HIFs transcriptional activity in trophoblasts would bring new perspectives on placenta-related diseases.

Angiogenesis is essential for the placental function and reproductive success. Circulating PGF is prominently elevated in pregnancy, promoting the development and maturation of the placental vascular system, but its bioavailability depends on sFlt-1 secretion that acts as a decoy receptor for PGF.[66] The regulation of sFlt-1:PGF under hypoxia was not fully elucidated. It had nevertheless been suggested that HIF-2α could inhibit *PGF* expression.[67] In BeWo cells, Sasagawa *et al.* had further demonstrated that HIF-2α mediates an hypoxia-induced upregulation of *FLT1* expression.[68] Our study extends these findings to human cytotrophoblasts and underlines the potential benefit of inhibiting HIF-2α on the angiogenic balance.

Whilst there has been little progress in prophylaxis, there are still no treatments for placental dysfunctions in normal obstetric practice. Hopefully, a recent surge in the field aims at repurposing drugs and therapies developed for other conditions as potential treatments for placental dysfunction.[69] For example, Brownfoot *et al.* demonstrated *in vitro* that metformin can reduce sFlt-1 secretion by inhibiting HIFs, but, to our knowledge, no treatment selectively targeting HIF-2α has been tested so far.[70] Interestingly, specific HIF-2α inhibitors have been developed for clear cell renal carcinoma.[71,72] In light of our results, such inhibitors should be considered in preclinical obstetrical settings.

Conclusively, using clinical samples and human primary cytotrophoblasts, we provide a molecular explanation to pathologies associated to placental hypoxia, where HIF-2α plays a key role, whereas HIF-1α does not. A main perspective to our work is to test selective HIF-2α inhibitors in preclinical models of FGR and/or PE.[73]

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TABLES

Table 1. Maternal and fetal characteristics

Data are presented as mean ± SD. **P < 0.01, ***P < 0.001 *versus* control, using Student's t-test.

	Control (11)	FGR (12)	PE (8)
Age (years)			
• Median	33	32	30.5
• Interquartile range	31-37	29.25-37	27.5-32.25
Ethnic group			
• Asian	1	1	0
• Black	2	1	0
• Mixed	1	3	1
• White	7	7	7
Body-mass index			
• Median	21	22	26
• Interquartile range	20-22.5	20.75-24.5	26-29
Obstetrical history			
• Nulliparous	2	4	5
• Multiparous with FGR	1	0	1
• Multiparous with PE	0	0	1
Gestational age at delivery (week.day)			
• Median	38.1	39.2	30.5
• Interquartile range	37.2-38.4	38.4-40.3	28.2-32
Mode of delivery			
• Vaginal	10	12	2
• C-section	1	0	6
Fetal sex			
• Female	6	6	2
• Male	5	6	6
Fetal weight (g)	3236.36 ± 497.88	2633.33 ± 318.64 (**)	1165 ± 726.37 (***)

Table 2. Histologic characterization of pathological placentas according to the Amsterdam Placental Workshop Group Consensus Statement

Data are presented as mean ± SD. *P < 0.05, ***P < 0.001 *versus* control, using Student's t-test

	Control (11)	FGR (12)	PE (8)
General			
• Placental weight (g)	423.75 ± 71.47	366.19 ± 57.13 (*)	215.56 ± 56.08 (***)
• Anomaly			
Placental shape	1	3	2
Membranes	0	0	1
• Umbilical cord diameter (mm)	13.27 ± 3.17	12.5 ± 2.02	10.25 ± 2.19
Maternal Vascular Malperfusion			
• Placental hypoplasia (< P10)	1	6	3
• Infarction	1	8	6
< 10%	1	7	4
> 10%	0	1	2
• Distal villous hypoplasia	2	7	5
• Accelerated villous maturation	1	5	5
Syncytial knots > 33%	3	8	8
Fibrin deposit	3	4	7
• Decidual arteriopathy	0	0	4
Fetal Vascular Malperfusion			
• Thrombosis	0	0	0
• Avascular villi	0	0	0
• Intramural fibrin deposition	0	0	0
• Stem vessel obliteration	0	0	0
• Vascular ectasia	2	4	4
Delayed Villous Maturation	0	3	0
Ascending Intrauterine Infection			
• Maternal Inflammatory Response	0	2	1
• Fetal Inflammatory Response	0	0	1
Villitis of Unknown Origin			
• Low	2	2	
• High	0	0	1
• Undetermined	1	0	0
Chorangiomatosis	1	1	0

FIGURES

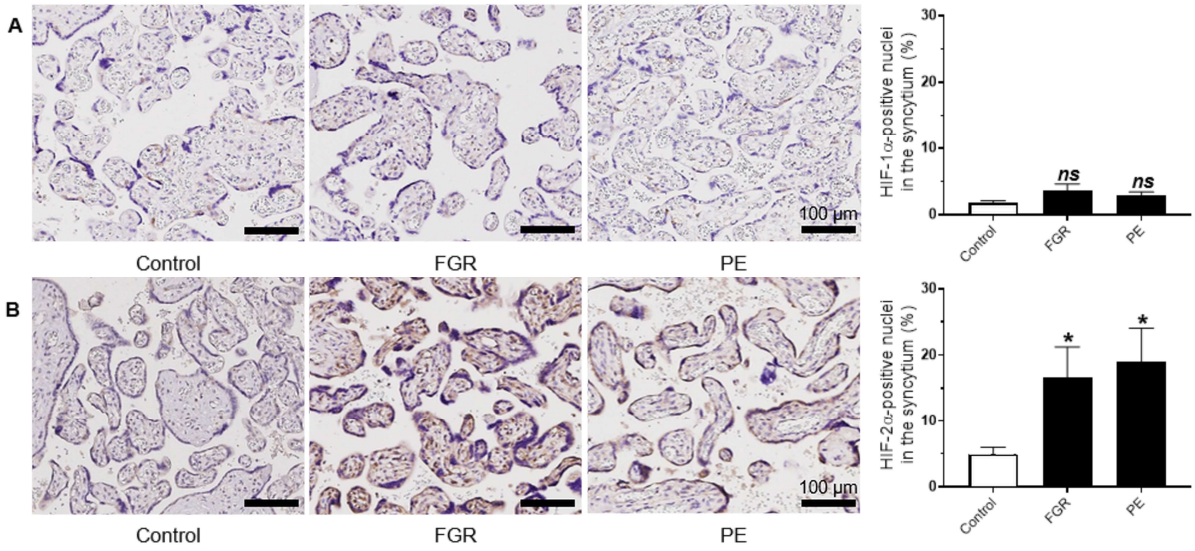

Figure 1. The nuclear expression of HIF-2α is increased in human pathological placentas from FGR or preeclampsia patients

The nuclear expression of **A**, HIF-1α and **B**, HIF-2α in the syncytium of normal human placentas (Control, *N* = 11, *n* = 66) and pathological human placentas from patients with fetal growth restriction alone (FGR, *N* = 12, *n* = 72) or associated to preeclampsia (PE, *N* = 8, *n* = 48) was quantified using immunostaining (brown) and haematoxylin counterstain**.** Graphs represent the percentage of positive nuclei in the syncytium determined by computer-assisted image analysis. *ns P* > 0.05; **P* < 0.05 compared to control, using one-way ANOVA with a Dunnett's multiple comparisons test (A-B). Scale bars, 100 µm.

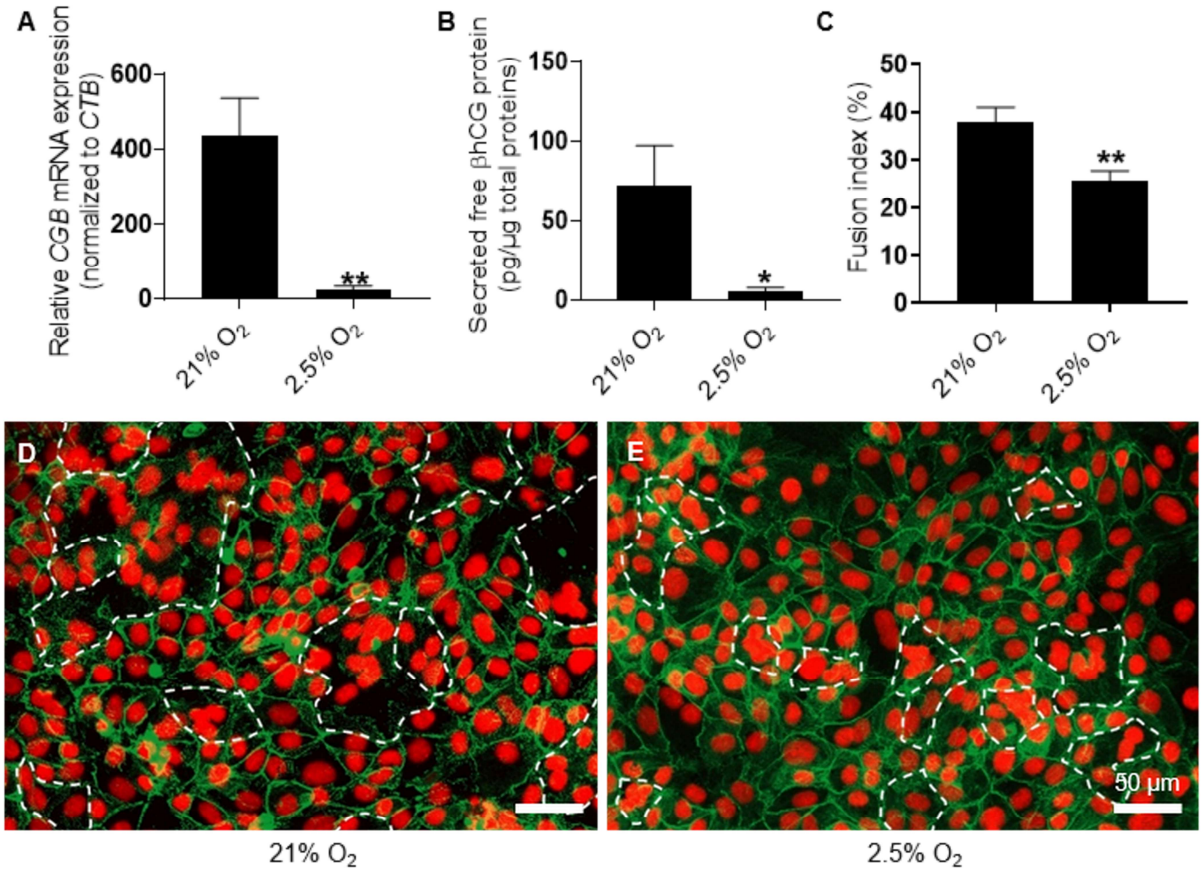

Figure 2. Hypoxia impairs the formation of the syncytium

Effects of the oxygen tension on human primary cytotrophoblast differentiation was studied after 5 days of culture under 21% O_2 or 2.5% O_2. *N* = 5 (*n* = 10) human placentas at term. **A**, *CBG* gene expression and **B**, secreted β subunit of human chorionic gonadotrophin (βhCG) were measured as markers of biochemical differentiation. **C,** The fusion index was manually calculated using membrane immunostaining. Pictures are representative of each condition (*N* = 5 human placentas, *n* = 30 fields per condition). Scale bars, 50 µm. All data are normalized to undifferentiated cytotrophoblasts (time 0). **P* < 0.05; ***P* < 0.01, using Student's t-test (A-C).

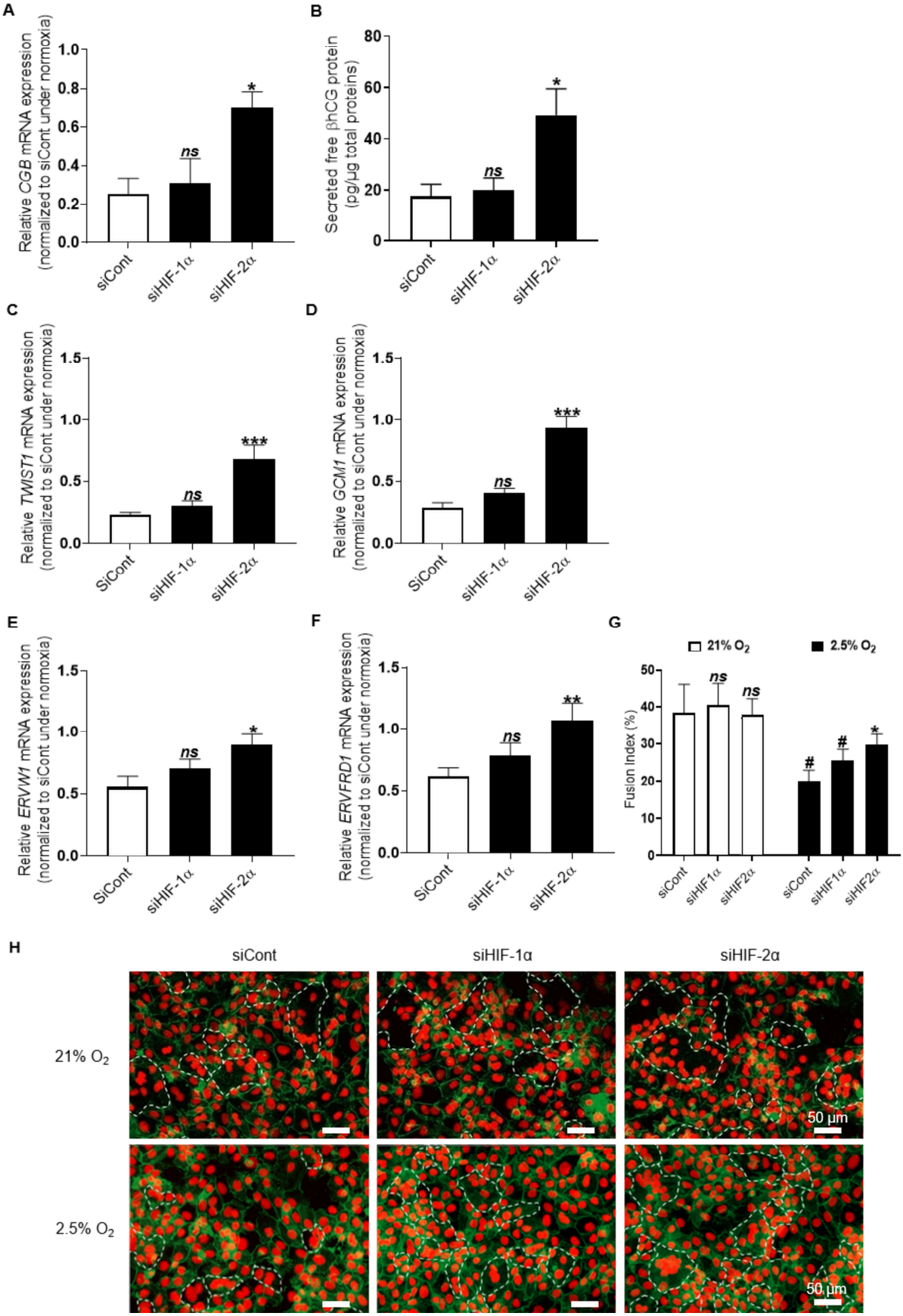
A
Relative CGB mRNA expression (normalized to siCont under normoxia)
1.0
0.8
0.6
0.4
0.2
0.0
ns
*
siCont
siHIF-1α
siHIF-2α
B
Secreted free βhCG protein (pg/µg total proteins)
80
60
40
20
0
ns
*
siCont
siHIF-1α
siHIF-2α
C
Relative TWIST1 mRNA expression (normalized to siCont under normoxia)
1.5
1.0
0.5
0.0
ns
***
SiCont
siHIF-1α
siHIF-2α
D
Relative GCM1 mRNA expression (normalized to siCont under normoxia)
1.5
1.0
0.5
0.0
ns
***
SiCont
siHIF-1α
siHIF-2α
E
Relative ERVW1 mRNA expression (normalized to siCont under normoxia)
1.5
1.0
0.5
0.0
ns
*
SiCont
siHIF-1α
siHIF-2α
F
Relative ERVFRD1 mRNA expression (normalized to siCont under normoxia)
1.5
1.0
0.5
0.0
ns
**
SiCont
siHIF-1α
siHIF-2α
G
21% O2
2.5% O2
Fusion Index (%)
50
40
30
20
10
0
ns
ns
#
#
*
siCont
siHIF1α
siHIF2α
siCont
siHIF1α
siHIF2α
H
siCont
siHIF-1α
siHIF-2α
21% O2
2.5% O2
50 µm
50 µm

Figure 3. HIF-2α inhibits human cytotrophoblast differentiation and fusion under hypoxia

Human primary cytotrophoblasts were infected with control siRNA (siCont) or with a cocktail of siRNAs specifically targeting either HIF-1α (siHIF-1α) or HIF-2α (siHIF-2α). Cells were then cultured for 5 days under 21% O_2 or 2.5% O_2. *N* = 5 (*n* = 10) human placentas at term. **A**, Relative (hypoxia:normoxia) *CBG* gene expression and **B**, secreted βhCG were measured as markers of biochemical differentiation. **C**, The relative (hypoxia:normoxia) expression of *TWIST1* was determined using RT-qPCR. **D**, same as A but for *GCM1*. **E**, Same as A but for *ERVW-1*/syncytin-1. **F**, Same as A but for *ERVFRD-1*/syncytin-2. **G-H**, The fusion index was manually calculated using membrane immunostaining. Pictures are representative of each condition (*N* = 5 human placentas, *n* = 30 fields per condition). Scale bars, 50 µm. *ns P* > 0.05, **P* < 0.05, ***P* < 0.01, ****P* < 0.005 *versus* the corresponding siCont using one-way ANOVA with a Dunnett's multiple comparisons test (A-E).; $^{\#}P$ < 0.05 *versus* the corresponding siRNA condition cultured under 21% using one-way ANOVA with a Dunnett's multiple comparisons test.

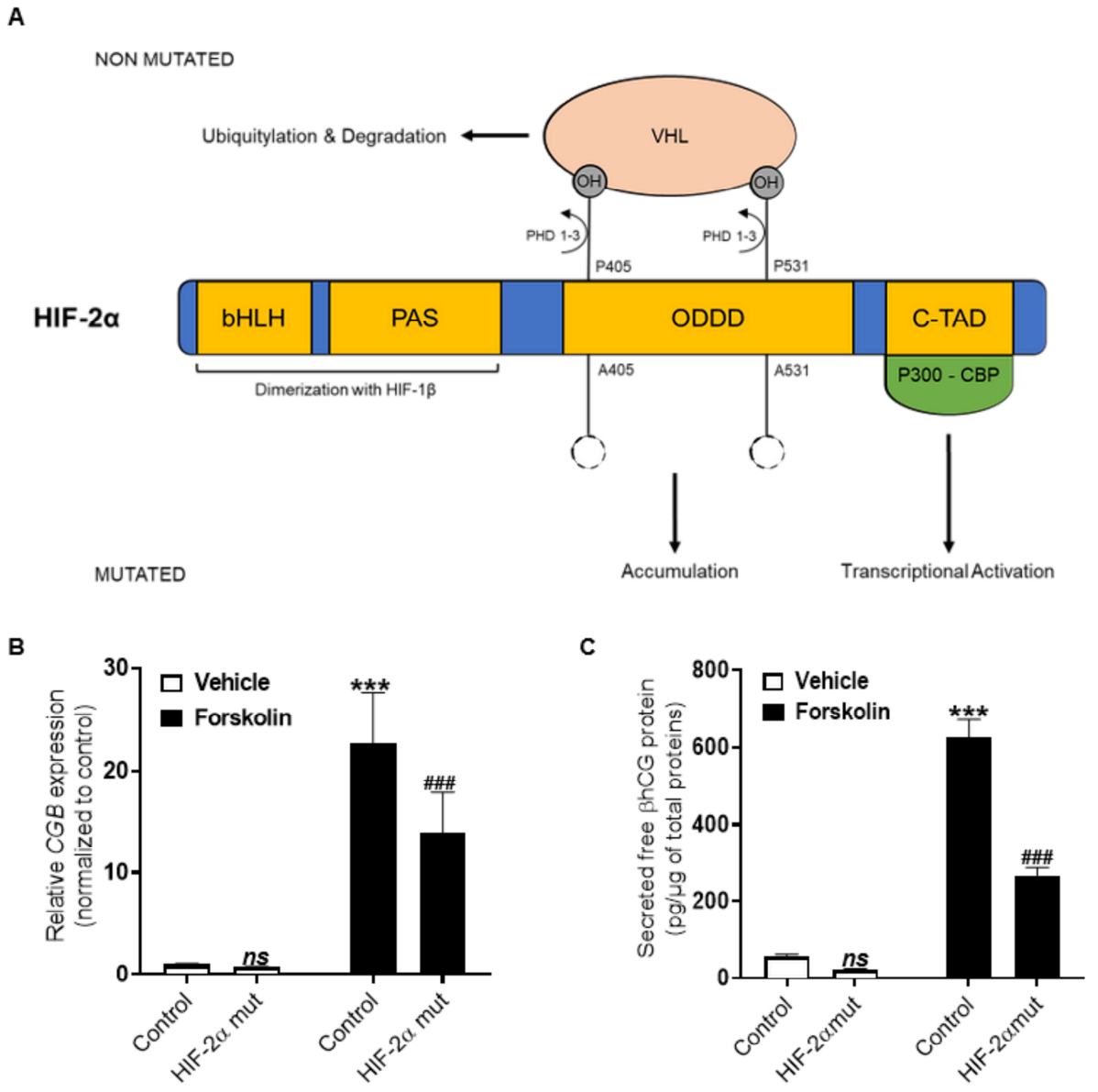

Figure 4. Experimentally induced overexpression of HIF-2α impairs BeWo cell syncytialization

A, Abnormally active human HIF-2α was generated by site-directed mutagenesis. Using mutagenic primers, prolyl residues were replaced by alanine residues in order to avoid PHD-mediated HIF-2α degradation. BeWo human choriocarcinoma cells were transfected with a vector encoding mutated HIF-2α (HIF-2α mut) or with an empty vector (Control). Cells were cultured under 21% O_2, and forskolin (25 µM for 24 h) was used to induce cell fusion where indicated (N = 2, n = 6). **B**, *CBG* gene expression and **C**, secreted βhCG were measured as markers of biochemical differentiation. *ns* $P > 0.05$, $^{***}P < 0.005$ *versus* control cells treated with vehicle; $^{\#\#\#}P < 0.005$ *versus* control cells treated with forskolin, using one-way ANOVA with a Tukey's multiple comparisons test.

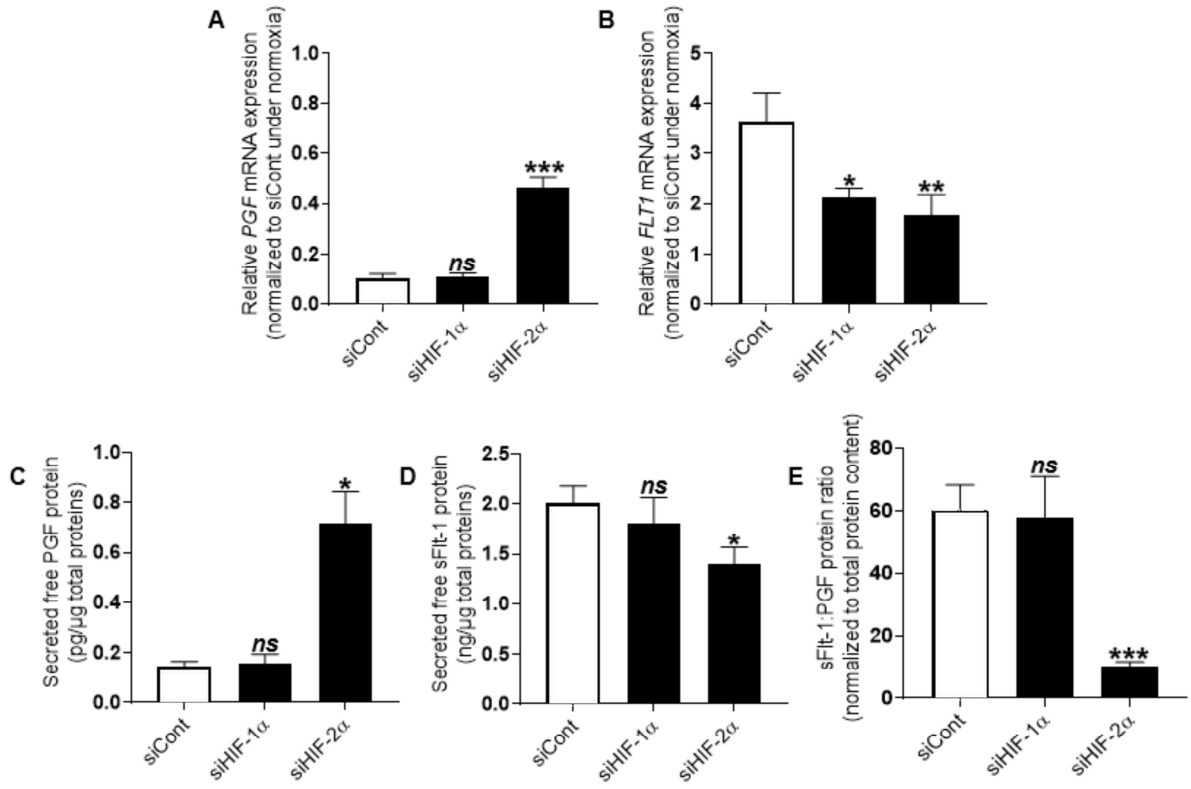

Figure 5. HIF-2α accounts for the decreased bioavailability of placental growth factor induced by hypoxia

Human primary cytotrophoblasts were transfected with control siRNA (siCont) or with a cocktail of siRNAs specifically targeting either HIF-1α (siHIF-1α) or HIF-2α (siHIF-2α). Cells were then cultured for 5 days under 21% O_2 or 2.5% O_2. *N* = 5 (*n* = 10) human placentas at term. **A**, The relative (hypoxia:normoxia) mRNA expression of placental growth factor (*PGF*) was quantified using RT-qPCR. **B**, Same as in A but for soluble decoy receptor *FLT1*. **C**, Secreted free PGF was measured in the cell supernatant of the last 24 h by ELISA and normalized to total protein content. **D,** Same as in C but for sFlt-1. **E,** The graph shows the sFlt-1:PGF ratio calculated based on ELISA protein quantification for each sample. *ns P* > 0.05, **P* < 0.05, ***P* < 0.01, ****P* < 0.005 *versus* siCont, using one-way ANOVA with a Dunnett's multiple comparisons test (A-E).