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Differential regulation of human PIGF gene expression in trophoblast and nontrophoblast cells by oxygen tension

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Abstract

Objective—To determine the mechanism for differential effects of low oxygen tension on human PIGF gene transcription in trophoblast and nontrophoblast cells.

Study Design—Human PIGF reporter clones and real time RT-PCR were used to compare the effects of hypoxia on gene transcription in human trophoblast and nontrophoblast cell lines. Overexpression of HIF-1 α , inhibition of HIF-1 function and biochemical assessments of HIF-1 co-factor interactions were used to characterize hypoxia response mechanisms regulating PIGF transcription.

Results—PIGF transcription is specifically inhibited by low oxygen tension in trophoblast but is induced in some nontrophoblast cells. Overexpression of HIF-1 α in normoxic cells or inhibition of HIF-1 function in hypoxic cells did not significantly alter transcription patterns of the PIGF gene in either cell type.

Conclusions—These results suggest that transcriptional repression of PIGF gene expression occurs in human trophoblast exposed to low oxygen tension but that PIGF transcription is stimulated in certain hypoxic nontrophoblast cells. However, regulation of PIGF transcription is not mediated by functional HIF-1 activity in either cell types.

Keywords

gene expression; hypoxia; hypoxia response element; placenta growth factor; pregnancy; trophoblast

Introduction

PIGF is an angiogenic growth factor both in vitro and in vivo [1]. Under normal physiological conditions, human PIGF expression in vivo is largely restricted to the placenta [2]. Within the human maternal-fetal interface, PIGF is prominently expressed in villous cytotrophoblast and syncytiotrophoblast [3;4] and uNK cells [5]. Previous studies from our laboratory and others

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have shown that maternal serum PlGF levels are significantly reduced in preeclampsia [6;7] and that this decrease occurs before clinical onset of the symptoms of preeclampsia [8;9]. In addition, presence of an alternatively spliced, soluble form of the PlGF receptor (sflt-1 or sVEGFR1) is increased in preeclamptic women [9]. Evidence from animal models highlight the functional importance of PlGF expression and bioavailability during pregnancy. Overexpression of ectopic sflt-1, which inhibits both VEGF and PlGF bioavailability, but not KDR, which antagonizes VEGF activity, results in maternal hypertension and proteinuria during pregnancy [10]. Furthermore, chronic maternal hypoxia decreases systemic levels of PlGF, increases sflt-1 levels, and results in significant intrauterine growth restriction [11]. Although viable, pups lacking PlGF expression have significantly reduced placental and fetal birth weights [12]. Collectively, these studies suggest decreased production of trophoblast PlGF coupled with decreased bioavailability of PlGF likely contribute to the placental, vascular, and renal pathologies commonly associated with preeclampsia [13].

Preeclampsia is thought to be associated with reduced placental perfusion and relative hypoxia [14]. Previous studies have confirmed that preeclamptic trophoblast express less PlGF mRNA than normal trophoblast [15]. In agreement with these *in vivo* studies, we [16] and others [17-19] have documented that hypoxia down regulates trophoblast PlGF gene expression *in vitro*. In contrast, PlGF mRNA expression is increased in many other cell types under low oxygen tension [20-24] and is differentially regulated by hypoxia inducible factor-1 (HIF-1) *in vivo* [25] suggesting cell type specific mechanisms of regulation.

HIF-1 is a key regulator of gene transcription in response to hypoxic conditions. In hypoxic conditions HIF-1 α protein is stabilized and dimerizes with HIF-1 β forming a functional HIF-1 heterodimer which binds to hypoxia response elements (HRE) within genes. Recruitment of the transcriptional co-activator p300/CREB binding protein (CBP) to HIF-1 forms a hypoxia-induced, HRE-bound complex, which is critical for transactivation of numerous genes, including vascular endothelial growth factor (VEGF) and erythropoietin [26].

Although putative hypoxia response elements have been identified in the human PlGF 5'UTR [20], the molecular mechanisms by which hypoxia differentially regulates human PlGF expression is not clear. Using reporter constructs of the human PlGF gene, we report that, in contrast to the effects of hypoxia on PlGF expression in other cells, hypoxia suppresses transcription of PlGF in trophoblast. We further demonstrate that regulation of PlGF transcription under hypoxic conditions is independent of HIF-1, despite putative consensus HREs within the 5'UTR of human PlGF gene.

Methods

Clone preparation

A 1.5 Kb region (-1521/+34) and 2 subregions (-1521/-650, -698/+34) of the human PlGF gene cloned into a β -galactosidase (β -gal) reporter vector pBlue-TOPO® (Invitrogen, Carlsbad, CA) have been previously described [27]. The promoter clone (-1521/+34) and distal subclone (-1521/-650) both contain two consensus HREs (5'TACGTG3') [26;28] at -1274 and -952 which is lacking in second proximal subclone (-698/+34) used in the present study (Figure 1). The pGL2-TK-HRE plasmid containing a minimal TK promoter fragment linked to a triple repeat consensus HRE (5'-GTGACTACGTGCTGCCCTAG-3') and a firefly luciferase reporter cassette was a generous gift from Dr. Giovanni Melillo [29]. Plasmids encoding constitutively expressed HIF-1 α (pCEP4/HIF-1 α) and a dominant negative form of HIF-1 α (pCEP4/HIF-1 α dn), were obtained from ATCC (Manassas, VA).

Cell culture

JEG-3 (choriocarcinoma), HeLa (cervical carcinoma), and hEK-293 (human embryonic kidney) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Fisher Scientific, Hanover Park, IL) supplemented with 10% fetal bovine serum (FBS) and (50µg/ml) penicillin G and streptomycin (Atlanta Biologicals, Lawrenceville, GA). Primary trophoblast were isolated from normal term placentae and cultured as described [30]. Use of primary human trophoblast was approved by the Institutional Review Board at Southern Illinois University School of Medicine. Hypoxic conditions were accomplished as described [16] or with 100µM of deferoxamine mesylate (DFO) (Sigma Aldrich, St. Louis, MO).

Transient transfections

Cells were co-transfected with 1.5µg of the PIGF clones (-1521/+34, -1521/-650, or -698/+34) with ExGen 500 (MBI Fermentas, MD) as described [27]. Normalization of each experiment was achieved by co-transfection (0.5µg) of an RSV driven firefly luciferase plasmid (RSV-Luc) (gift from Dr. Stuart Adler).

To assess the hypoxia responsiveness of each cell line, 1.5µg of the pGL2-TK-HRE clone was co-transfected with 0.5µg of a pSV40-β-gal control vector (Promega, Madison, WI). Experiments utilizing 1.0µg pCEP4/HIF-1α or pCEP4/HIF-1αdn expression vectors incorporated 0.75µg of the (-1521/+34) PIGF clone and 0.25µg RSV-Luc. 1.0µg of the pCEP4 backbone vector (Invitrogen) was used to bring the total amount of transfected DNA up to 2µg and to control for possible cross-promoter activities between co-transfected plasmids [31]. Transfected cells were exposed to 1% O₂ for indicated time points. Control cultures were maintained in atmospheric oxygen conditions (21% O₂) [16]. Protein cell lysates were prepared, and β-gal and luciferase activities were determined using a Dual Light Kit (Applied Biosystems, Foster City, CA) according to instructions. Relative light units were measured in a Beckman Coulter LD 400C Luminescence Detector (Fullerton, CA).

Transfection Data Analyses

β-gal activity of each PIGF-reporter construct was normalized to the co-transfected luciferase activity as described [27] and relative differences between culture conditions (hypoxia vs. normoxia) and time (24 vs. 48hrs) were determined. Relative differences in luciferase based reporter clones were determined as described above but normalized to co-transfected β-gal activities. All experiments were performed in duplicate for all conditions with data expressed as mean ± SEM. A one-sample Student *t*-test was used to calculate fold increase or decrease of the experimental clones. A *p* value ≤ 0.05 was considered statistically significant.

PIGF mRNA Real-Time PCR

Changes in PIGF mRNA expression were detected by reverse transcribing 500ng-1µg of total RNA according to instructions (iScript cDNA Synthesis kit, Bio-Rad, Hercules, CA.). 2µl of the RT reaction was added to iQ SYBR Green Supermix containing 0.1µM of PIGF primers that amplify a 150 bp cDNA region common to all known isoforms of human PIGF [32]. Specificity, validity, and efficiency of the reaction were confirmed by gel electrophoresis and melting curve analyses. Control reactions included primers for RPL32, a ribosomal associated protein that is stable in hypoxic conditions [33], to control for RNA integrity and PCR efficiency. Relative changes in transcript levels between untreated control (a) and test samples (b) was calculated using the following formula: $2^{[(Ct(a)-CtRPL32(a))-(Ct(b)-CtRPL32(b))]}$ [34]. The amount of PIGF mRNA derived from untreated control cultures served as the baseline (set to 100%).

Nuclear Protein Extraction and Western blot analysis

JEG-3, HeLa, and hEK-293 cells were exposed to 21% O₂ or 1% O₂ conditions for 24 hours. Cells were lysed and nuclear and cytoplasmic fractions were prepared according to instructions (Active Motif Nuclear Extract Kit, Carlsbad, CA). Protein concentrations were determined using D_c Protein Assay (Bio-Rad, Hercules, CA). Extraction of nuclear proteins were confirmed by probing for Lamin A/C (BD Biosciences, San Diego, CA) and only extractions indicating a presence of Lamin A/C in nuclear but not cytoplasmic extracts were used for analyses (data not shown). Equal amounts of protein were separated on 7.5% SDS-PAGE gels, transferred to nitrocellulose membranes and immunoblotted with antibodies against HIF-1 α (BD Biosciences) overnight at 4°C. Chemiluminescence detected using ECL (Amersham, Piscataway, NJ). Lamin A/C expression fluctuates under changing O₂ conditions (data not shown) and therefore could not serve as a loading control. Since β -actin is expressed in the nuclear compartment [35] and did not fluctuate under hypoxia, HIF-1 α expression was normalized to β -actin signals. The protein bands were quantified using an automated digital densitometry software program (UN-SCAN IT, Silk Scientific Inc., Orem, UT).

HRE co-immunoprecipitation

HIF-1 binding to consensus hypoxia response element (HRE) was performed as described [36] with minor modifications. Briefly, equimolar concentrations of a biotinylated 54-mer oligonucleotide corresponding to a triple repeat of the functional HRE 5' GCCCTACGTGCTGTCTCA 3' [37] were annealed. Contained within this oligonucleotide is the minimally functional HRE sequence 5'TACGTG 3' [36;38] which is repeated twice within the 1.5 Kb region of the PIGF promoter (Figure 1). Biotinylated double-stranded HRE was bound to Avidin-D matrix (Vector Laboratories, Burlingame, CA), and combined with 70 μ g of nuclear extract from either normoxic or hypoxic cells as described previously [27]. Avidin-D beads were re-suspended in 2X Laemmli sample buffer and proteins in the supernatants immunoblotted for HIF-1 α and p300 (Upstate, Charlottesville, VA).

Results

Functional capabilities of various PIGF promoter clones in trophoblast cells have been shown [27]. In experiments duplicated for this report, both the (-1521/+34) clone and the proximal clone (-698/+34), but not a distal clone (-1521/-650), produced significant promoter activity ($p < 0.01$) by 24 hrs in JEG-3 cells. Sustained transcriptional activity of the promoter constructs was evidenced by significant increases in reporter activity of the 1.5Kb PIGF clone (5.7 ± 1.0 fold; $p < 0.05$, $n=6$) and proximal (-698/+34) clone (3.4 ± 0.2 fold; $p < 0.01$, $n=6$) under normoxic conditions between 24 and 48 hour time points in JEG-3 cells (data not shown). No significant transcriptional activity was observed for distal clone (-1521/-650) in any cell type nor under any culture condition. For clarity and brevity, we do not include this distal clone in the figures for this report.

Transfected JEG-3 cells were subjected to 1% O₂ to determine the effect of low oxygen tension on transcriptional activity of the PIGF promoter regions in trophoblast cell lines (Figure 2A). Transcriptional activity of the (-1521/+34) clone was reduced by 3.4 ± 0.78 fold ($p < 0.01$) after 24 hours and by 11.3 ± 2.5 fold ($p < 0.01$) after 48 hours in hypoxia when compared to 21% O₂. Similarly, activity of the proximal (-698/+34) clone was reduced 2.26 ± 0.36 fold ($p < 0.01$) after 24 hours and 10.1 ± 1.8 fold ($p < 0.01$) after 48 hours in hypoxia. These results establish that hypoxia significantly decreases transcriptional activity of the PIGF promoter in trophoblast cells.

To investigate cell type specific effects of hypoxia on PIGF expression in nontrophoblast cells, transfection experiments were performed in HeLa cells (Figure 2B). The (-1521/+34) clone

demonstrated a significant 3.93 ± 1.0 fold ($p < 0.02$) increase in transcriptional activity after 24 hours of hypoxia. In contrast, the (-698/+34) clone did not produce a significant increase in transcriptional activity (Figure 2B) consistent with the lack of consensus HRE sites in this clone. These results are in sharp contrast to those in trophoblast, and indicate a cell type specific hypoxic regulation of PIGF transcription.

Real time RT-PCR confirmed cell type specific effects of low oxygen tension on endogenous PIGF mRNA accumulation in non-transfected cells (Figure 2C). In agreement with the PIGF promoter reporter gene experiments, hypoxia significantly decreased PIGF mRNA expression in JEG-3 cells and primary trophoblast. In contrast, hypoxia significantly increased PIGF mRNA in HeLa cells. Collectively, these results confirm that hypoxia can selectively alter PIGF mRNA in cells and that the decrease in trophoblast is mediated, at least in part, by an inhibition of transcription. Thus, we undertook experiments to determine functional differences in HIF-1 mechanics between trophoblast and nontrophoblast cells.

Failure of the consensus HRE sites to mediate an increase in PIGF gene transcription in trophoblast could reflect a lack of adequate HIF-1 α production and translocation to the nucleus. However, hypoxia induced a 4.4 ± 0.013 fold increase ($p < 0.05$) in HIF-1 α protein accumulation in the nuclei of hypoxic primary trophoblast and similar increases in nuclear HIF-1 α expression were detected in JEG-3 and both nontrophoblast cells lines, hEK-293 and HeLa (Figure 3). HIF-1 α was undetectable in cytoplasmic extracts from any of the cells (data not shown). Although HIF-1 α stabilization and translocation to the nucleus was evident in primary trophoblast and JEG-3 cells, other possibilities include HIF-1 binding to the PIGF HREs and/or recruitment of essential co-activator proteins (p300/CBP) [26] are deficient in trophoblast. One prominent regulator of HIF-1 functional activity within the nucleus is CITED-2 [39]. Expression dynamics of CITED-2 and the effects of hypoxia on its expression in human trophoblast are not known. Temporally, expression of PIGF mRNA was variably decreased by 3 hrs and continued to decrease to 30% of control levels by 6hrs to 10% by 24 hrs of hypoxia (Figure 4A). In contrast, expression of CITED-2 mRNA was largely unchanged by 3hrs of hypoxia but increased by 20% at 6hrs and 50% by 24 hrs of hypoxia. Increased CITED-2 expression could competitively limit p300/CBP recruitment to PIGF HRE-HIF-1 complexes in trophoblast at extended time points. However, HRE oligo-binding assays demonstrated similar patterns of nuclear HIF-1 α binding to a consensus HRE oligomer and p300 recruitment to the HRE/HIF-1 α complex in hypoxic JEG-3, hEK-293, and HeLa cells (Figure 4B). No HIF-1 α nor p300 proteins were co-precipitated by the HRE oligo from nuclear lysates of cells maintained in 21% O₂. Interestingly, in primary trophoblast we could not detect HIF-1 α binding to the consensus HRE after 24 hours in hypoxia (data not shown). This finding is surprising given that down regulation of PIGF mRNA (Figure 2C) is consistent between JEG-3 cells and primary trophoblast; however, the amount of HIF-1 α detected in hypoxic primary trophoblast nuclei is notably less than in an equal amount of nuclear lysate for JEG-3 cells (see Figure 3A). Although relatively similar nuclear HIF-1/p300 complex formation occurs between the cell lines, functional activity of HIF-1/p300 complexes may be different.

Transcriptional activity of theHRE reporter (pGL2-TK-HRE) significantly increased under hypoxic conditions in all three cell lines (JEG-3 = 14.2 ± 4.1 fold, hEK-293 = 19.7 ± 4.6 fold, HeLa = 17.2 ± 6.7 fold) (Figure 4C), and further increased at 48 hrs under hypoxia (data not shown). These results suggest little differences in hypoxia-induced transcription and HRE-mediated responses between the cells.

Induction of HIF-1 α is usually associated with increased expression of genes containing HREs. However, specific transcriptional repression by HIF-1 α is known to occur in some genes that contain putative HRE sequences [40]. We overexpressed constitutively active or dominant negative HIF-1 α in JEG-3 and HeLa cells to determine functional requirements for HIF-1 α in

regulating the transcription of PIGF. No endogenous HIF-1 α protein was detectable in nuclear extracts of JEG-3 under standard (21% O₂) conditions; however, HIF-1 α protein was evident in nuclear extracts of JEG-3 cells at 1% O₂ and in cells transfected with the HIF-1 α expression clone at 21% O₂ (Figure 5A). Similar results were obtained in HeLa cells (data not shown). Overexpression of HIF-1 α did not affect transcriptional activity of the 1.5Kb PIGF promoter in JEG-3 cells under 21% O₂ (Figure 5B). Similarly, overexpression of HIF-1 α in HeLa cells, where activity of the PIGF 1.5Kb promoter is increased under hypoxia (see Figure 2C), had no significant effect under normoxic conditions (Figure 5B). Increasing concentrations of HIF-1 α expression clone also produced no significant effect on transcriptional activity of the PIGF reporter clone in either cell type (data not shown). However, overexpression of HIF-1 α induced significant increases (3-5 fold) in transcriptional activity of the HRE-reporter construct in 21% O₂ in both JEG-3 and HeLa cells (Figure 5C).

Inhibiting HIF-1 α functional activity during 1% O₂ exposure with dominant negative HIF-1 α (pCEP4/HIF-1 α dn) did not affect hypoxia responsiveness of the PIGF reporter in HeLa cells (Figure 5B). Furthermore, inhibition of HIF-1 α activity in hypoxic trophoblast did not restore activity of the PIGF promoter in JEG-3 cells (Figure 5B). However, overexpression of dominant negative HIF-1 α significantly reduced activity of the HRE reporter construct in both cell types under 1% O₂ (Figure 5C). Collectively, these results confirm that PIGF transcription is differentially regulated by oxygen in trophoblast versus nontrophoblast cell types and highlights that this regulation is independent of HIF-1 functional activity in the cell types studied.

Discussion

The human PIGF gene is highly expressed in normal trophoblast, but expression is reduced in pathological conditions such as preeclampsia. Although it is established that low oxygen tension differentially reduces PIGF mRNA expression in trophoblast [16-19] while increasing PIGF expression in many nontrophoblast cell types [20;22-24], the involvement of HIF-1 mediated mechanisms behind this regulation is not clear. We have incorporated select regions of the 5' upstream region of the human PIGF gene to investigate oxygen regulation and the direct role of HIF-1 α in mediating PIGF transcription in trophoblast and nontrophoblast cells.

Functional activity mediated by both the -1.5 Kb and -698 bp regions of human PIGF gene was significantly decreased by low O₂ tension in JEG-3 cells. These results indicate that the hypoxia-induced decrease in PIGF mRNA expression is mediated at least in part by a significant decrease in transcription. The -1.5 Kb promoter clone containing 2 consensus HRE motifs produced increased reporter activity in nontrophoblast cells (HeLa) under hypoxia, which correlates well with increased PIGF mRNA expression observed in these cells. The hypoxia responsiveness of this clone, but not a clone lacking these motifs (-698/+34), suggested that differences in HIF-1 mediated activity between trophoblast and nontrophoblast may differentially regulate PIGF expression.

The lack of hypoxia responsiveness of the PIGF 5' UTR region in trophoblast does not appear to be due to defects in HIF-1 complex formation or functional activity. Comparable induction of HIF-1 α protein expression, nuclear translocation, and cognate HRE binding occurred in the trophoblast and nontrophoblast cells. Although similar HIF-1 levels were detected, there are several key regulatory points that govern HIF-1 functional activity within the nucleus. Such mechanisms involve members of the CITED protein family, particularly CITED-2 and CITED-4, have been shown to compete for p300/CBP co-activator binding to HIF-1 and functionally restrict HIF-1 mediated activity [41]. We found that CITED-2 mRNA expression increased in hypoxic trophoblast in a temporal pattern that was inversely related to the decrease in PIGF mRNA expression in the same cells. However, we detected similar recruitment of p300

to HIF-1/HRE complexes in trophoblast and nontrophoblast cell lines under hypoxic culture conditions. Furthermore, a consensus HRE reporter construct indicated that trophoblast respond similarly in magnitude as nontrophoblast to hypoxic insult. Collectively, these results argue against a cell-type specific defect in oxygen sensing or deficiencies in HIF-1 α mediated responses between the different cell types.

Hypoxia stimulated gene transcription is synchronized through numerous interacting transcription factor binding sites and multiple protein complexes. These adjacent transcription factors and multi-protein complexes formed in response to hypoxia may be unique to each responsive gene [36]. A possible explanation for specific down regulation of PlGF transcription in hypoxic trophoblast could be that other transcription factors binding to sequences adjacent to the PlGF HREs may be sequestering the hypoxic induction of PlGF. For example, when three functional HREs within the lactate dehydrogenase A (LDH-A) gene promoter were concatemerized, high level hypoxic induction was not evident indicating that each HRE is essential but not sufficient for hypoxic response[36]. Potential effects of the surrounding sequences in human PlGF gene and transcription factors binding to these sequences need to be addressed.

Little is known about transcriptional regulation of PlGF gene expression in any cell type. Recently, we have shown that PlGF transcriptional activity is mediated in part by glial cell missing-1 (GCM1) transcription factor in trophoblast [27]. Similarly, metal transcription factor-1 (MTF1) has recently been shown to regulate PlGF expression in trophoblast [42]. Both of these transcription factors are decreased in hypoxic trophoblast in vitro and in preeclamptic trophoblast in vivo [27;42;43]. Thus, it is likely that decreases in GCM1 and/or MTF1 activity during hypoxia may lead to decreased PlGF expression in trophoblast. The effects of hypoxia on PlGF gene expression in nontrophoblast is cell type and/or condition specific in that expression can be induced by overexpression of HIF-1 α in some, but not all cells [25]. Over expression of NF κ B p65 can increase PlGF mRNA accumulation in hEK293 cells and can cooperate with MTF1 to further increase expression levels [21]. However, overexpression of MTF1 did not increase PlGF mRNA expression in normoxic hEK293 cells, and although hypoxia resulted in increased nuclear translocation of NF κ B and MTF in the cells, expression of PlGF mRNA was not increased [21]. Hypoxia did not increase PlGF mRNA expression in human aortic or umbilical vein endothelial cells in vitro [21]. In contrast, PlGF gene transcription is increased in hypoxia stressed, Ras-transformed mouse fibroblasts but is attenuated in cells lacking MTF1 [20]. Other studies have shown that MTF1 and HIF-1 α may associate to form a transcriptional complex to mediate hypoxia-induced expression of metallothionein-1 gene [44]. We found that induction of PlGF gene transcription is not mediated by HIF-1 driven factors since overexpression of a dominant negative HIF-1 α could not inhibit hypoxia-induced PlGF expression in HeLa cells. Furthermore, despite consensus HRE sites and accumulation of functional nuclear HIF-1/p300 complexes, PlGF transcription was inhibited in hypoxic trophoblast and inhibition of HIF-1 activity could not restore PlGF expression in these cells. Collectively, these results highlight the complex regulation of PlGF gene transcription in different cells and further emphasize the cell type specific regulation of PlGF gene expression.

Preeclamptic placentae are characterized by decreased invasion of endovascular trophoblast into the spiral arteries of the endometrium which is thought to lead to local placental bed hypoxia with elevated protein levels of HIF-1 α [45]. We have shown that hypoxia decreases PlGF gene transcription, albeit through mechanisms independent of HIF-1, which may provide a causal link to the observed decrease in PlGF associated with preeclampsia. Our evidence suggests that decreased expression of PlGF mRNA in hypoxic trophoblast is due, at least in part, to a decrease in PlGF gene transcription. Our data reaffirms that regulation of PlGF

transcription is cell type specific and further studies to characterize the molecular nature of this unique regulation may provide targeted avenues for therapeutic interventions in preeclampsia.

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Figure 1. Schematic of PIGF Promoter Clones

Specific regions of the 5' flanking region of the human PIGF gene were PCR amplified, ligated into pBlueTOPO and verified by bidirectional sequencing and alignment with the published human genome sequence for each region as detailed previously [27]. H = consensus HIF-1 response elements (HRE).

A. **B.**

C.

Figure 2. Differential effects of oxygen tension on PIGF transcription and mRNA expression in trophoblast versus nontrophoblast

(A) Hypoxia significantly decreases transcription of PIGF promoter clones in trophoblast. JEG-3 cells were co-transfected with RSV-luc and PIGF promoter clones (-1521/+34) or (-698/+34). Fold decrease of β -gal activity under 1% O₂ conditions from β -gal activity in 21% O₂ is plotted ($\pm$ SEM). All decreases in transcriptional activity were statistically significant (* = $p < 0.01$) from 21% O₂ levels ($n \geq 5$ independent experiments). (B) Hypoxia increases transcription of (-1521/+34) PIGF clone in HeLa cells. Cells were co-transfected as in panel A and fold increase in β -gal activity under 1% O₂ conditions compared to 21% O₂ normoxic values plotted, * = $p < 0.02$, $n = 9$ independent experiments. (C) Cell type specific effects of hypoxia on PIGF mRNA expression. Primary trophoblast, JEG-3, and HeLa cells were cultured under 21% O₂ or 1% O₂ for 24hrs. PIGF mRNA expression was determined by real-time RT-PCR. Data is plotted as mean percent change from normoxic culture conditions (21% O₂ = 100%), * = $p \leq 0.05$.

Figure 3. Hypoxia induces similar HIF-1 α expression and nuclear translocation between cell types (A). Cells were subjected to culture conditions at 21% O₂ or 1% O₂ for 24 hrs. 40 μ g of nuclear protein lysates were prepared and subjected to Western blot analysis for HIF-1 α . Membranes were stripped and re-probed for β -actin to normalize for protein loading. (n = 3).

C

Figure 4. Hypoxia induces CITED-2 expression and similar HIF-1 α translocation, p300 recruitment, and functional HIF-1 activity

(A). Hypoxia induces CITED-2 mRNA expression and decreases PlGF expression in trophoblast. JEG-3 cells were subjected to DFO (3hr) or 1% O₂ for 6 and 24hrs. Relative change in mRNA expression for each time point was determined by real-time RT-PCR and normalized to levels at 21% O₂ (set = 1). (B) Nuclear HIF-1 α in hypoxic cells binds to consensus HRE and recruits p300. Nuclear protein lysates were mixed with Avidin-D-HRE beads and proteins bound to the HRE-oligo were subjected to Western blot analysis for p300 and HIF-1 α . Representative blots from 5-7 independent assays are shown. (C) Hypoxia induces similar HIF-1 functional activity in trophoblast and nontrophoblast cell lines. Cells were co-transfected with pSV- β -gal and pGL2-TK-HRE, and cultured in 21% O₂ or 1% O₂ conditions. Fold increase of luciferase activity under hypoxic conditions above activity in 21% O₂ is plotted, n = 6 independent experiments/cell line, p<0.05 from normoxic values for each cell line.

A.

B.

Figure 5. Transcriptional activity of PIGF promoter (-1521/+34) under normoxic or hypoxic cell culture conditions is independent of HIF-1 α

(A.) JEG-3 cells were transfected with pCEP-4/HIF-1 α , subjected to 21% O₂ or 1% O₂ for 24hrs, and nuclear proteins immunoblotted for HIF-1 α and β -actin. (B and C.) JEG-3 and HeLa cells were co-transfected with PIGF clone (-1521/+34) (panel B) or pGL2-TK-HRE (panel C) reporter clone and either pCEP-4, pCEP-4/HIF-1 α or pCEP-4/HIF-1 α dn. Cells were lysed after 24 hrs in 21% O₂ or 1% O₂ conditions and normalized percent change in reporter activities from control transfections plotted with * = < 0.05 (n=5).