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# Differential Regulation of Human PlGF 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 *PlGF* gene transcription in trophoblast and nontrophoblast cells.
*Study design:* Human PlGF 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-1α, inhibition of HIF-1 function and biochemical assessments of HIF-1 co-factor interactions were used to characterize hypoxia response mechanisms regulating *PlGF* transcription.
*Results:* PlGF transcription is specifically inhibited by low oxygen tension in trophoblast but is induced in some nontrophoblast cells. Overexpression of HIF-1α in normoxic cells or inhibition of HIF-1 function in hypoxic cells did not significantly alter transcription patterns of the *PlGF* gene in either cell type.
*Conclusions:* These results suggest that transcriptional repression of *PlGF* gene expression occurs in human trophoblast exposed to low oxygen tension but that *PlGF* transcription is stimulated in certain hypoxic nontrophoblast cells. However, regulation of *PlGF* transcription is not mediated by functional HIF-1 activity in either cell type.



## 1. Introduction

Placenta growth factor (PlGF) is an angiogenic growth factor both in vitro and in vivo [1]. Under normal physiological conditions, human PlGF expression in vivo is largely restricted to the placenta [2]. Within the human maternal–fetal interface, PlGF is prominently expressed in villous cytotrophoblast and syncytiotrophoblast [3,4] and uNK cells [5]. Previous studies from our laboratory and others 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-1α protein is stabilized and dimerizes with HIF-1β forming a functional HIF-1

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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.

## 2. Methods

### 2.1. 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 (Fig. 1). The pGL2-TK-HRE plasmid containing a minimal TK promoter fragment linked to a triple repeat consensus HRE (5′-GTGACTACGTGCTGCCTAG-3′) and a firefly luciferase reporter cassette was a generous gift from Dr. Giovanni Melillo [29]. Plasmids encoding constitutively expressed HIF-1α (pCEP4/HIF-1α) and a dominant negative form of HIF-1α (pCEP4/HIF-1αdn), were obtained from ATCC (Manassas, VA).

### 2.2. 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 (50 μg/ml) penicillin G and streptomycin (Atlanta Biologicals, Lawrenceville, GA). Primary cytotrophoblast cells 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 100 μM of deferoxamine mesylate (DFO) (Sigma Aldrich, St. Louis, MO).

### 2.3. Transient transfections

Cells were co-transfected with 1.5 μg of the PlGF 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 (0.5 μg) of an RSV driven firefly luciferase plasmid (RSV-Luc) (gift from Dr. Stuart Adler).

To assess the hypoxia responsiveness of each cell line, 1.5 μg of the pGL2-TK-HRE clone was co-transfected with 0.5 μg of a pSV40-β-gal control vector (Promega, Madison, WI). Experiments utilizing 1.0 μg pCEP4/HIF-1α or pCEP4/HIF-1αdn expression vectors incorporated 0.75 μg of the (−1521/+34) PlGF clone and 0.25 μg RSV-Luc. 1.0 μg of the pCEP4 backbone vector (Invitrogen) was used to bring the total amount of transfected DNA up to 2 μg and to control for possible cross-promoter activities between co-transfected plasmids [31]. Transfected cells were exposed to 1% $O_2$ for indicated time points. Control cultures were maintained in atmospheric oxygen conditions (21% $O_2$) [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).

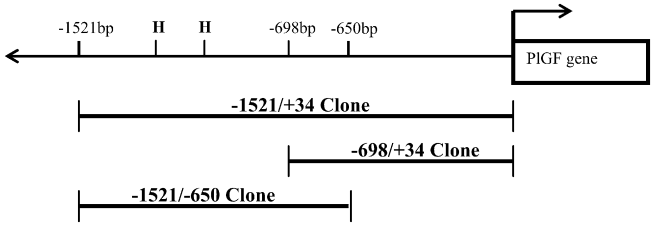


**Fig. 1.** Schematic of PlGF Promoter Clones. Specific regions of the 5′ flanking region of the human *PlGF* gene were PCR amplified, ligated into pBlueTOPO and verified by bidirectional sequencing and alignment with the published human genome sequence for each region as detailed previously [27]. H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

### 2.4. Transfection data analyses

β-gal activity of each PlGF 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. 48 h) 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's *t*-test was used to calculate fold increase or decrease of the experimental clones. A $p$ value $\leq 0.05$ was considered statistically significant.

### 2.5. PlGF mRNA real-time PCR

Changes in PlGF mRNA expression were detected by reverse transcribing 500 ng to 1 μg of total RNA according to instructions (iScript cDNA Synthesis kit, Bio-Rad, Hercules, CA). 2 μl of the RT reaction was added to iQ SYBR Green Supermix containing 0.1 μM of PlGF primers that amplify a 150-bp cDNA region common to all known isoforms of human PlGF [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) were calculated using the following formula: $2^{[(Ct(a)-CtRPL32(a))]-[(Ct(b)-CtRPL32(b))]}$ [34]. The amount of PlGF mRNA derived from untreated control cultures served as the baseline (set to 100%).

### 2.6. Nuclear protein extraction and Western blot analysis

JEG-3, HeLa, and hEK-293 cells were exposed to 21% $O_2$ or 1% $O_2$ conditions for 24 h. 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 was 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-1α (BD Biosciences) overnight at 4 °C. Chemiluminescence detected using ECL (Amersham, Piscataway, NJ). Lamin A/C expression fluctuates under changing $O_2$ 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-1α 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).

### 2.7. 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 PlGF promoter (Fig. 1). Biotinylated double-stranded HRE was bound to Avidin-D matrix (Vector Laboratories, Burlingame, CA), and combined with 70 μg of nuclear extract from either normoxic or hypoxic cells as described previously [27]. Avidin-D beads were re-suspended in 2× Laemmli sample buffer and proteins in the supernatants immunoblotted for HIF-1α and p300 (Upstate, Charlottesville, VA).

## 3. Results

Functional capabilities of various PlGF 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 h in JEG-3 cells. Sustained transcriptional activity of the promoter constructs was evidenced by significant increases in reporter activity of the 1.5 kb PlGF 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 h time points in JEG-3 cells (data not shown).