Molecular Regulation of Human Placental Growth Factor (PIGF) Gene Expression in Placental Villi and Trophoblast Cells is Mediated via the Protein Kinase A Pathway

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Abstract

Cyclic 3',5'-adenosine monophosphate (cAMP) is a critical second messenger for human trophoblasts and regulates the expression of numerous genes. It is known to stimulate *in vitro* the fusion and differentiation of BeWo choriocarcinoma cells, which acquire characteristics of syncytiotrophoblasts. A DNA microarray analysis of BeWo cells undergoing forskolin-induced syncytialization revealed that among the induced genes, placental growth factor (PIGF) was 10-fold upregulated. We verified this result in two choriocarcinoma cell lines, BeWo and JEG-3, and also in first trimester placental villous explants by quantifying PIGF mRNA (real time PCR) and PIGF protein secreted into the supernatant (ELISA). Similar effects were noted for vascular endothelial growth factor (VEGF) mRNA and protein expression. Treatment with cholera toxin and the use of a specific inhibitor of protein kinase A (PKA) blocked these effects, indicating that the cAMP/PKA pathway is responsible for the cAMP-induced upregulation of PIGF and that one or more G protein coupled receptor(s) was involved. We identified two functional cAMP responsive elements (CRE) in the PIGF promoter and demonstrated that the CRE binding protein, CREB, contributes to the regulation of PIGF gene expression. We speculate that defects in this signaling pathway may lead to abnormal secretion of PIGF protein as observed in the pregnancy-related diseases preeclampsia and intrauterine growth restriction.

Keywords

cAMP, angiogenesis, choriocarcinoma

Introduction

The placenta is critical to the intrauterine life of eutherian mammals and has at least 2 essential functions. It is a miniature endocrine system producing and secreting hormones, cytokines, and vasoactive growth factors affecting maternal metabolism and fetal growth. It also provides a physical interface, protecting the conceptus yet allowing nutrient and gas exchange between the maternal and fetal blood circulations. In the human and other hemochorial species, this close contact between the 2 circulations is achieved via densely vascularized fetal villi floating in maternal blood that fills the intervillous space. The surface of the villi is covered with polynucleated cells called syncytiotrophoblasts that arise from the fusion and differentiation of cytotrophoblast stem cells. These syncytiotrophoblasts, which are in direct contact with the maternal blood, are the primary site of production and secretion of proangiogenic and antiangiogenic factors like vascular endothelial growth factor A (VEGF-A), placental growth factor (PIGF), and the soluble form of their common receptor sVEGFR1. These factors simultaneously regulate fetal blood

vessel formation in the placenta as well as maternal vascular function during the course of pregnancy.¹

Hormones and small peptides are also important in the regulation of the vascular function. Estrogens and progesterone modulate vascular tone during pregnancy and mediate aspects of maternal cardiovascular adaptation²; however in the setting of menopausal hormone therapy, these sex steroids are also linked to risks of venous thromboembolism.^{3,4} Likewise, the renin-angiotensin-aldosterone system plays an important role in vascular remodeling during pregnancy and has been associated with arterial hypertension and preeclampsia (PE).^{5,6}

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Prominent among a variety of second messenger molecules that link hormone action with cell signaling is cyclic 3',5'-adenosine monophosphate (cAMP). Cyclic AMP modulates the expression of many trophoblast gene products and regulates a spectrum of growth and differentiation pathways in human placenta. Its biosynthesis classically is stimulated by binding of a ligand to G-protein-coupled membrane receptors (GPCRs), with subsequent activation of adenylate cyclase, the regulatory subunit of protein kinase A (PKA), and ultimately phosphorylation of the transcription factor cAMP-responsive element-binding protein (CREB). Phosphorylated CREB homodimerizes and binds to cAMP-responsive elements (CREs) located in the promoters of its target genes, leading to the initiation of their transcription.

The cAMP-PKA-CREB pathway has been implicated in several networks of human placental gene activation, including those involved in endocrine differentiation of trophoblasts. Choriocarcinoma cells (BeWo) provide a very good in vitro model to study human trophoblast differentiation. When they are treated for 72 hours with either cAMP or forskolin, a strong activator of adenylate cyclase, BeWo cells fuse and form polynucleated cells representing differentiated characteristics of syncytiotrophoblasts.^{7,8} This fusion process is under the regulation of syncytin, a gene coding for an envelope protein of retroviral origin.⁹ While the pathways are not fully elucidated, cAMP, through PKA, upregulates the expression and the transcriptional activity of the placenta-specific transcription factor, glial cells missing 1 (GCM1),¹⁰ which binds to the syncytin gene promoter and upregulates its transcription.¹¹ Several other trophoblast genes are also upregulated by cAMP, including those encoding the α and β subunits of human chorionic gonadotropin (hCG) and aromatase (hCYP19).^{12,13} These genes promote hCG and estrogen production.

Placental angiogenesis, which can be detected in the human as early as 1 week postimplantation, continues until term. Several factors regulating placental angiogenesis are VEGF-A, PlGF, sVEGFR1, and soluble Endoglin (sEng). Alteration in the expression of these factors is associated with abnormal vascularization of the placental bed that is a hallmark of the pregnancy-related diseases, PE and intrauterine growth restriction (IUGR). In the serum of women with PE, total VEGF levels are reported to be increased¹⁴ while free PlGF levels are decreased^{15,16}; at the same time, the antiangiogenic factors sVEGFR1 and sEng are both increased.¹⁷ Given the important role of cAMP in the regulation of trophoblast differentiation, we postulated that it might also be involved in placental angiogenesis. For example, PlGF is a product of the syncytiotrophoblasts¹⁸ and its gene is 10-fold upregulated during differentiation of BeWo cells after treatment with forskolin, as revealed by complementary DNA (cDNA) microarray analysis.¹⁹ In human granulosa cells, VEGF is upregulated by cAMP through the PKA pathway.²⁰ In the human endometrium, VEGF also is upregulated by cAMP.²¹

To better understand human placental *PlGF* gene regulation, we investigated the effects of the cAMP/PKA pathway on *PlGF* gene expression and protein secretion in placental villous explants and 2 choriocarcinoma cell lines. Our findings

indicate that PKA is a potent regulator of *PlGF* gene expression and protein secretion in trophoblasts.

Materials and Methods

Drugs

Dibutyl-*c*-AMP (N6,2'-O-dibutyladenosine 3',5'-cyclic monophosphate sodium salt), cholera toxin from *Vibrio cholerae* and H-89 (dihydrochloride hydrate), a specific inhibitor of PKA (dihydrochloride hydrate) were purchased from Sigma-Aldrich (St Louis, Missouri). The adenylate cyclase activator forskolin (*Coleus forskolii*) was purchased from Calbiochem-EMD Chemicals, Inc (Gibbstown, New Jersey). Minimum essential medium (MEM) Eagle with Earle Balanced Salts Solution containing 50 μ g/mL gentamicin (Sigma) and 10% fetal bovine serum (FBS), Cellgro-Mediatech, Herndon, Virginia) were used.

Placenta Villous Explants

After written informed consent, placenta tissue was collected between 11 and 13 gestational weeks from voluntary pregnancy terminations, under institutional review board (IRB) approval from the University of California, San Francisco (UCSF) and Emory University School of Medicine. Fragments of villi were isolated under sterile conditions, washed with phosphate buffered saline (PBS) without calcium and magnesium, and minced in MEM. Resuspended villi were placed into suspension culture overnight at 37°C in humidified incubators equilibrated with 95% air (20% oxygen) and 5% CO₂.

Cell Cultures

JEG-3 (HTB-36) and BeWo (CCL-98) cells were purchased from American Type Culture Collection (ATCC, Manassas, Virginia). JEG-3 cells were grown in MEM, supplemented with 50 μ g/mL gentamicin and 10% fetal calf serum. BeWo cells were grown in Kaighn F12K medium (ATCC) supplemented with 50 μ g/mL gentamicin and 10% FBS.

Total RNA Extraction/Purification

The explants or cells were treated for 8 to 18 hours with the different drugs and total RNAs were extracted using the PureLink Micro-to-Midi total RNA Purification System (Invitrogen, Carlsbad, California).

Reverse Transcription and Quantitative Real-Time Polymerase Chain Reaction

Total tissue or cellular RNA (0.5 μ g) was used as template for reverse transcription in 20 μ L reaction volumes, using iScript cDNA synthesis kit (Bio-Rad, Hercules, California) then diluted 5 times with RNase-free water.

Two litre cDNA and 0.6 μ mol/L specific primers, as listed in Table 1 for human PlGF, RNA polymerase II (RPII) and

Table 1. Primers for Real-Time PCR Analysis of mRNA Encoding Human PIGF and Internal Controls RNA Polymerase II and GAPDH

Gene		Primer Sequences	Amplicon Size
PIGF	Sense	5'-CAGAGGTGGAAGTGGTACCCTTCC-3'	223 bp
	Antisense	5'-CGGATCTTTAGGAGCTGCATGGTGAC-3'	
RNA Pol II	Sense	5'-GCACCACGTCCAATGACAT-3'	269 bp
	Antisense	5'-GTGCGGCTGCTTCCATAA-3'	
GAPDH	Sense	5'-GAGTCAACGGATTGGTCGT-3'	184 bp
	Antisense	5'-GACAAGCTTCCCGTTCTCAG-3'	

Abbreviations: PIGF, placental growth factor; PCR, polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; mRNA, messenger RNA.

glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were added to iQTM SYBR Green supermix (Bio-Rad). The polymerase chain reaction (PCR) amplification was performed on a DNA Engine Opticon 2 system (Bio-Rad) using the following thermal protocol: 95°C, 3 minutes followed by 40 cycles of a denaturation step at 95°C for 30 seconds, a hybridization step at 61°C for 30 seconds, and signal reading. Polymerase chain reactions were performed in triplicate for each cDNA, averaged, and normalized to RPII or GAPDH reference transcripts. The specificity of the amplification was determined by doing a melting curve (55°C–95°C, held and read every 10 seconds at each 0.5°C increment) which generated sharp, single-product peaks for each transcript. RNA was quantified using the Ct method of relative quantification using a Microsoft Excel Macro spreadsheet designed for Gene Expression Analysis for iCycler iQ Real-Time PCR Detection System (Bio-Rad).

Enzyme-Linked Immunosorbent Assay

PIGF and VEGF protein concentrations, from JEG-3 and BeWo conditioned media were measured using commercially available quantitative sandwich enzyme-linked immunosorbent assays ([ELISAs] R&D Systems, Minneapolis, Minnesota).

Reporter Genes and Expression Vectors

A 3450-bp PIGF promoter was amplified by PCR from genomic DNA using primers with KpnI recognition sequences added to the forward primer and BglII sequences added to the reverse primer. The amplified DNA was gel-purified with QIAquick gel extraction kit (QIAGEN, Germantown, Massachusetts), digested with KpnI/BglII (Promega, Madison, Wisconsin), and cloned into the luciferase reporter plasmid pGL3 basic (Promega). A KpnI site was inserted by site-directed mutagenesis (QuickChange XL Site-Directed Mutagenesis Kit, Stratagene, La Jolla, California), to create 2141-, 1253-, 905-, 478-, and 304-bp PIGF fragments and positive clones were digested with KpnI. The fragments were gel-purified and religated. Glyceraldehyde 3-phosphate dehydrogenase–renilla luciferase reporter constructed in the same pGL3 basic plasmid was used to normalize transfection efficiency. Expression vectors pCMV-CREB (overexpression) and pCMV-KCREB (dominant negative) were purchased from Clontech Laboratories, Inc (Palo Alto, California).

Transfection

JEG-3 cells were seeded at a density of 1.5×10^5 cells per well in 12-well plates for 24 hours and transfected with 1 µg PIGF promoter reporter plasmid and 10 ng GAPDH-renilla plasmid (internal control) per well using FuGene 6 (Roche, Indianapolis, Indiana). After 24 hours, the medium was removed and replaced by fresh 10% FBS medium and treated with the drugs for 8 hours at the final concentrations indicated in the figures. Luciferase activity was assayed using the Dual-Luciferase Reporter Assay System (Promega).

Electromobility Shift Assays

Nuclear extracts from JEG-3 cells treated for 30 minutes with 10 µmol/L forskolin were prepared following the NE-PER kit protocol (Pierce, Rockford, Illinois). The double-stranded oligonucleotides –40/+1 containing the 3 wild-type putative CRE sites (underlined, 5'-CATGAGCCTTGACGGCTGACGCTGGAC-3') were purchased from Operon Biotechnologies (Huntsville, Alabama) and labeled at the 5' end with [32 P]ATP by T4 polynucleotide kinase. Mutated competitor probes M1 (5'-CATtataaaTGACGGCTGACGCTGGAC-3' [lower case nucleotides indicate mutated bases]), M2 (5'-CATGAGCCTTtataaaTGACGCTGGAC-3'), M3 (5'-CATGAGCCTTGACGGCTtataaaGGAC-3'), M4 (5'-CATtataaaTtataaaTGACGCTGGA C-3'), M5 (5'-CATtataaaTGACGGCTtataaaGGAC-3'), M6 (5'-CATGAGCCTTtataaaTtataaaGGAC-3'), and M7 (CATtataaatta aaTtataaaGGAC-3') also were obtained from Operon Biotechnologies.

Nuclear extract, 10 µg, were incubated for 10 minutes on ice in the binding reaction buffer containing 3.33 mmol/L 1,4-dithiothreitol, 10 µg bovine serum albumin (BSA), 1 µg poly (dI-dC), 100 mmol/L Tris-HCl pH 8; 300 mmol/L KCl, 5 mmol/L EDTA, and 10% glycerol. For the competition reactions, 1.75 pmol cold probes were added to the mixture and incubated 5 minutes at room temperature. Then, 0.035 pmol [32 P]probe was added and the reactions were incubated 30 minutes at room temperature. The DNA-protein complexes were separated at 4°C in a 4% acrylamide/bisacrylamide, 1× Tris-acetate-EDTA (TAE) buffer (40 mmol/L Tris, 20 mmol/L acetate, 1 mmol/L EDTA) gel at 200 V in 1× TAE running buffer. The complexes were visualized by autoradiography.

Figure 1. The effect of cAMP/PKA pathway modulators on PIGF mRNA expression in first trimester human placental villous explants. First trimester placental villous tissue was minced and cultured overnight in MEM and treated with vehicle (CTL), 1 mmol/L dbcAMP (cAMP), 10 μ mol/L forskolin (Fsk) or 100 ng/mL cholera toxin (ChTx). Total RNA was extracted and PIGF transcripts were quantified by RT-qPCR, normalized to RNA polymerase II as a constitutive control, and expressed relative to control conditions. cAMP indicates cyclic 3',5'-adenosine monophosphate; PKA, protein kinase A; PIGF, placental growth factor; MEM, minimum essential medium; RT-qPCR, reverse transcription and quantitative real-time polymerase chain reaction.

Statistical Analysis

Each experiment was repeated a minimum of 3 times, with the results normalized to control conditions for statistical analyses. The data, which were normally distributed by Kolmogorov-Smirnov tests, are expressed as mean $\pm$ standard error and were compared using analysis of variance (ANOVA) and Student *t* tests. Two-tailed probabilities <0.05 were considered statistically significant.

Results

Cyclic AMP Upregulates PIGF Messenger RNA Expression in First Trimester Placenta Villous Explants and 2 Choriocarcinoma Cell Lines

We analyzed the expression of PIGF messenger RNA (mRNA) using real-time PCR in placental villous explants. Glyceraldehyde 3-phosphate dehydrogenase and RNA polymerase II cDNAs were used as internal controls, with the latter proving to be more consistent, as reported previously in placenta.²² Overnight treatment of villous explants obtained from first trimester placenta with 1 mmol/L dibutyryl (db-) cAMP (a cell-permeable analog) increased PIGF mRNA expression >2 -fold (Figure 1). To verify the activation of the cAMP-PKA pathway,

we treated the villous explants overnight with 10 μ mol/L forskolin (a strong activator of adenylate cyclase) or with 100 ng/mL cholera toxin (which activates stimulatory G α protein). Forskolin and cholera toxin induced 3-fold and >2 -fold increases in PIGF mRNA compared to control, respectively.

We extended the same experiments using JEG-3 and BeWo cells, which express characteristic placental hormones,²³ human leukocyte antigen (HLA)-G²⁴, and nuclear receptors,²⁵ and are well characterized models of human trophoblasts. The cells were treated for 8 hours with 1 mmol/L db-cAMP, 10 μ mol/L forskolin or 100 ng/mL cholera toxin (Figure 2A and B). In JEG-3 cells, cAMP induced a 7-fold increase in PIGF mRNA expression compared to control. Forskolin and cholera toxin each upregulated PIGF expression ≥ 9 -fold. Placental growth factor mRNA upregulation by forskolin and cholera toxin was inhibited by 50% and 60%, respectively, in the presence of 10 μ mol/L of the PKA inhibitor H-89.

The findings in BeWo cells were identical. Cyclic AMP, forskolin, and cholera toxin had similar effects, but these were less potent, increasing PIGF mRNA expression by 2-, 4-, and >3 -fold, respectively.

Cyclic AMP/PKA Signaling Pathway Increases PIGF Protein Secretion From JEG-3 and BeWo cells

To verify that changes in gene expression were reflected by protein synthesis, supernatants from cells treated with the same compounds were subjected to a specific PIGF ELISA (Figure 2C and D). In JEG-3 cells, cAMP, forskolin, and cholera toxin increased PIGF protein by 3-, 5-, and >5 -fold, respectively, after 24 hours. As noted at the mRNA level, H-89 inhibited both forskolin and cholera toxin-induced PIGF secretion by $\geq 60\%$.

Again, responses in BeWo cell-conditioned medium paralleled those in JEG-3 cells but with less amplitude. Cyclic AMP, forskolin, and cholera toxin increased PIGF protein secretion ~ 2 -fold and H-89 inhibited forskolin-induced PIGF secretion by $\sim 30\%$.

Vascular endothelial growth factor protein secretion was also measured. The effects of cAMP, forskolin, and cholera toxin are more subtle but the overall trend was the same (data not shown).

A 3.45-kb PIGF Promoter Reporter is Upregulated by Forskolin in JEG-3 Cells

We amplified 3.45 kb of the human *PIGF* gene flanking sequence promoter using primers designed to add KpnI and BglII sites at the 5' and 3' ends, respectively, of the promoter. This KpnI/BglII PIGF construct was cloned into a promoterless luciferase reporter plasmid (pGL3 basic) and transfected into JEG-3, followed by treatment for 8 hours with 1 mmol/L db-cAMP, 10 μ mol/L forskolin, 100 ng/mL cholera toxin, 10 μ mol/L H-89 alone or in combination (Figure 3). Cyclic AMP, forskolin, and cholera toxin induced 3-, 5- and 7-fold

Figure 2. The effects of cAMP/PKA pathway modulators on PIGF mRNA expression in 2 choriocarcinoma cell lines, JEG-3 (panel A) and BeWo (panel B) and on PIGF protein secretion into the supernatant of these same cell lines JEG-3 (panel C) and BeWo (panel D). Cells were grown under standard culture conditions for 24 hours and then treated for 8 hours with vehicle (CTL), 1 mmol/L dbcAMP (cAMP), 10 μ mol/L forskolin (Fsk), 100 ng/mL cholera toxin (ChTx), 10 μ mol/L H-89 or a combination of 10 μ mol/L Fsk/10 μ mol/L H-89 or 100 ng/mL ChTx/10 μ mol/L H-89. Supernatants were analyzed for free PIGF protein by ELISA and cells were used for RNA extraction, cDNA synthesis, purification, and RT-qPCR with PIGF-specific primers. cAMP indicates cyclic 3',5'-adenosine monophosphate; PKA, protein kinase A; PIGF, placental growth factor; mRNA, messenger RNA; RT-qPCR, reverse transcription and quantitative real-time polymerase chain reaction; cDNA, complementary DNA; ELISA, enzyme-linked immunosorbent assay.

increases, respectively, in promoter activation, whereas these effects were completely inhibited by H-89.

Cyclic AMP-Responsiveness is Localized in a 304-bp PIGF Promoter Fragment

To localize regions of the promoter with cAMP responsiveness, we systematically deleted sequences from the 5' end of the 3.45-kb PIGF promoter and subcloned these fragments to create PIGF promoter luciferase plasmids with the respective sizes of 2141, 1253, 905, 478, and 304 bp. JEG-3 cells were transfected for 24 hours with these reporter plasmids and treated with 10 μ mol/L forskolin for 8 hours (Figure 4). 5' deletion progressively decreased the luciferase gene activation from 6- to 2-fold, which was similar to the basal stimulation observed in the empty vector. However, the 304-bp promoter construct retained a strong response to forskolin with an 8-fold response in luciferase gene activation.

Computational analysis of the 304-bp PIGF promoter with MatInspector software (Genomatix, Munich, Germany)²⁶ identified 2 putative CREs immediately downstream from the TATA box. These CRE-like sequences consist of the 5 bases (underlined) corresponding to the core CRE consensus sequence TGACGTCA. Further analysis revealed the presence of a third motif that differs from the core CRE sequence only by the inverted positions of 2 bases (TGACG to TGAGC). We refer to these 3 potential CREs by numbers 1, 2, and 3 from 5' to 3' (Figure 5, left panel).

Electromobility Shift Assays Define 2 Functional CRE Motifs

To determine whether the CRE-like sequences were able to bind JEG-3 nuclear proteins, we performed electromobility shift assays (EMSAs). An intense band corresponding to the DNA-protein complex was observed when nuclear extracts

Figure 3. The effect of cAMP/PKA pathway modulators on PIGF promoter activity in choriocarcinoma JEG-3 cells. JEG-3 cells were transfected with 1 μ g of a PIGF promoter–firefly luciferase construct (containing 3.45 kb of PIGF promoter cloned into pGL3) and 10 ng of a GAPDH promoter–renilla luciferase construct. The cells were transfected for 24 hours and then treated with vehicle (CTL), 10 μ M/L H-89, 1 mmol/L dbcAMP, 10 μ M/L forskolin (Fsk), 100 ng/mL cholera toxin (ChTx) alone or in combination with H-89 for 8 hours. Dual-luciferase activity was then measured and fold activity above control was plotted. cAMP indicates cyclic 3',5'-adenosine monophosphate; PKA, protein kinase A; PIGF, placental growth factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

from untreated JEG-3 cells were incubated with a 27-bp probe containing all 3 wild-type CRE-like motifs (Figure 5). Thirty-minute pretreatment with forskolin prior to nuclear extract preparation did not increase the binding, consistent with reports that phosphorylation of CREB is not necessary for DNA binding.^{27,28}

The complex disappeared when a 50-fold excess of unlabeled wild-type probe was added to the reaction mixture (lane 4), indicating specificity. To evaluate the importance of each individual CRE sequence, the sites were independently mutated singly (M1, M2, and M3), doubly (M4, M5, and M6) or triply (M7). Competition experiments were performed by adding a 50-fold excess of the cold mutated probes to each labeled reaction. None of the mutated probes was able to compete except M2, in which the central CRE-like motif was mutated. This result demonstrated that CRE 2 is not bound by nuclear proteins.

Overexpression of wild-type CREB protein in JEG-3 cells only slightly increased forskolin-induced PIGF gene expression (Figure 6A). However, forskolin-induced PIGF gene expression was inhibited by 40% when a CREB-dominant negative protein was overexpressed. These results suggest that CREB plays a role in the cAMP-induced upregulation of PIGF gene, but it is apparent that other transcription factors are involved. A GCM1-binding site recently was identified within the 304-bp fragment of the human PIGF promoter and GCM1 gene and protein were reported to be upregulated by increases in intracellular cAMP.²⁹ We mutated the GCM1-binding site in

the 304-bp PIGF promoter, but this modification failed to inhibit the cAMP/forskolin-induced upregulation of PIGF promoter-driven luciferase gene expression (data not shown). These latter results argue against GCM1 as a transcription factor involved in cAMP upregulation of the PIGF gene.

Since we demonstrated by EMSA that CRE 1 and 3 sites were bound in vitro by nuclear proteins, we deleted a 40-bp fragment from the 304-bp PIGF promoter luciferase reporter plasmid that contained those CREs (Figure 6B). Deletion of the sites reduced the activation of the luciferase gene by forskolin by >50%. However, the observation that the deleted CREs did not fully abrogate forskolin activation indicates that other cAMP-responsive motifs must contribute to PIGF gene upregulation during trophoblast differentiation.

Discussion

Fusion and differentiation of trophoblasts occur through unknown mechanisms in vivo, but this process can be mimicked by treatment with cAMP or forskolin in vitro. BeWo choriocarcinoma cells can be induced to fuse under these conditions, which is associated with a corresponding upregulation of syncytin and hCG production. As expected, DNA microarray analysis of BeWo cells undergoing forskolin-induced syncytialization showed that the hCG β -subunit gene was 49-fold upregulated and the angiogenic growth factor PIGF was upregulated 10-fold.¹⁹

Our study confirmed that the PIGF gene was upregulated by cAMP in placental villous explants and in choriocarcinoma cell lines. Vascular endothelial growth factor production also was upregulated by cAMP, but its activation was subdued in comparison to PIGF gene expression. This latter result might explain why VEGF was not cited as upregulated significantly during syncytialization in the cDNA microarray studies of Kudo et al.¹⁹

Circulating levels of VEGF-A and PIGF proteins can be detected in the maternal serum as early as 8 weeks' gestation. Longitudinal studies measuring PIGF in the serum of pregnant women by ELISA show that free PIGF continues to increase during the course of pregnancy but starts to decline around 29 to 32 weeks' gestation. The control of its secretion remains unexplained.^{15,16}

Unlike the VEGF promoter which has been studied intensively, the organization and regulation of the human PIGF gene are only beginning to be characterized. Several nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B)-binding sites were identified in the human PIGF promoter,³⁰ and this transcription factor was invoked as a key regulator element. Overexpression of NF- κ B p65 in human embryonic kidney (HEK293) cells increased PIGF expression. In other studies, hypoxia synergistically increased NF- κ B p65-activated PIGF expression.

Another factor, GCM1, was shown to regulate PIGF gene transcription.²⁹ This transcription factor, originally identified in *Drosophila melanogaster*, is responsible for glial cell and macrophage formation.^{31–33} However, its critical role in the

Figure 4. Systematic 5' deletion of the PIGF promoter revealed that a 304-bp fragment retains a strong forskolin-induced luciferase activity. Five deletion constructs (2141, 1253, 905, 478, and 304 bp) were created from our 3.45-kb PIGF promoter—firefly luciferase (PIGFp) reporter. The sizes of the constructs were checked on an ethidium bromide-stained agarose gel and all plasmid sequences were confirmed. JEG-3 cells were transfected with 1 μ g of each PIGF construct and 10 ng GAPDH promoter—renilla luciferase plasmid for 24 hours prior to treatment with vehicle (CTL) or 10 μ mol/L forskolin (Fsk) for 8 hours. For each construct, normalized fold change above control was plotted. PIGF indicates placental growth factor.

Figure 5. The 304-bp PIGF promoter possesses 2 functional CRE-like motifs. Computational analysis of the wild-type 304-bp PIGF promoter fragment with MatInspector software defined 3 putative cAMP response element sites (left panel, "WT") located at the 3' end of the fragment. Electromobility shift assay (right panel) revealed that only site 1 and site 3 are bound by nuclear proteins extracted from JEG-3 cells. Both control (CTL) and forskolin (Fsk) treated cell nuclear extracts (NE) bound the [32 P]labeled oligonucleotides. Wild-type (WT) cold oligonucleotides and those mutated in site 2 (M2) were effective competitors of labeled oligonucleotide binding, whereas other mutations failed to compete for nuclear protein binding. The locations of the TATA box and GCM1-binding sites are also shown (left panel). PIGF indicates placental growth factor; cAMP indicates cyclic 3',5'-adenosine monophosphate; CRE, cAMP-responsive element; GCM1, glial cells missing 1.

Figure 6. CRE-like motifs and CREB are partly responsible for the cAMP effect on PIGF promoter. **A.** Expression vectors for wild-type CREB and a dominant negative CREB were transfected into JEG-3 cells for 24 hours. The cells were treated with vehicle or 10 $\mu\text{mol/L}$ forskolin for 8 hours. After RNA extraction and cDNA synthesis, PIGF cDNA was quantified by qPCR and normalized to the internal control RNA polymerase II gene, as described in the Figure 1 legend. Untreated (vehicle) cells transfected with mock vector were considered as control and results were calculated as fold change above control. Forskolin (Fsk) significantly stimulated 304 bp PIGF-luciferase expression in the presence of exogenous (mock vector) and overexpression of wild-type (wt) CREB ($*P < .05$) but not when dominant negative (dn) CREB protein was overexpressed. **B.** Deletion of the CRE-like motifs in 304 bp PIGF promoter–luciferase resulted in a >50% reduction in forskolin-induced PIGF/GAPDH luciferase activity ratio, relative to the wild-type promoter ($*P < .05$). PIGF indicates placental growth factor; cAMP indicates cyclic 3',5'-adenosine monophosphate; CRE, cAMP-responsive element; CREB, cAMP-responsive element-binding protein; cDNA, complementary DNA; qPCR, quantitative polymerase chain reaction.

development of the placenta was discovered when mice null for GCM1 were observed to die in utero around day 10 because of placental vascular failure.³⁴

The experiments described in our report demonstrate for the first time that cAMP is a strong activator of *PIGF* gene expression in human trophoblasts. We verified that this effect is mediated through activation of PKA. Other research indicates that the main target of PKA in the nucleus is CREB, which is phosphorylated on serine 133. This phosphorylation allows interaction with the coactivator CBP/p300 and recruitment of the ternary transcription complex.

We identified 2 functional CREs in the PIGF promoter which are partly necessary for cAMP activation of *PIGF* gene transcription. Overexpression of a CREB-dominant negative mutant significantly inhibited forskolin-induced upregulation

of *PIGF* gene expression (Figure 6A). The recently discovered GCM1-binding site lays approximately 230 bp upstream of the CRE motifs. We postulated that GCM1 might be a target of PKA, since it was previously shown that cAMP enhanced GCM1 transcriptional activity and increased its interaction with CBP/p300.³⁵ However, when we mutated this GCM1-binding site, we failed to observe any inhibition of the forskolin-induced PIGF promoter activity in JEG-3 cells (data not shown).

The activation of the cAMP/PKA-signaling pathway, mediated by membrane adenylate cyclase and G α stimulatory proteins, suggests that 1 or more GPCR is responsible for our observation. G-protein-coupled membrane receptors represent the largest class of cell surface receptors, comprising about 200 GPCRs with known ligands and 80 orphan GPCRs. Several

of these are expressed in the placenta and are under active investigation presently.

Interesting candidate ligands that could increase intracellular cAMP via GPCRs include calcitonin gene-related peptide (CGRP) and adrenomedullin (AM). Both molecules are expressed in the placenta and are known to have vascular effects.^{36,37} The 2 ligands share a common membrane receptor, the calcitonin receptor-like receptor (CRLR), which is associated with specific receptor activity modifying proteins (RAMPs) and the G-protein complex.

We previously observed low PIGF levels in the circulation of women destined to develop PE and IUGR¹⁵ and suggested that abnormal production of angiogenic factors like PIGF might be characteristic of pregnancy-related diseases.¹ Preeclampsia is characterized by hypertension and proteinuria and is associated with low levels of CGRP and CRLR mRNA in placental samples.³⁸⁻⁴¹ However, some reports indicate that AM mRNA and protein may be increased in the placenta and fetoplacental circulation of women with PE and IUGR.⁴²⁻⁴⁴

A more thorough comprehension of how PIGF is regulated during normal pregnancy is an important prerequisite to understanding its abnormal secretion, as observed in the serum of women developing pregnancy-related diseases like PE and IUGR. Reagents and models described in this report will be useful tools to ascertain *PIGF* gene regulation in human trophoblasts.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

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