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Glial Cell Missing 1 Regulates Placental Growth Factor (*PGF*) Gene Transcription in Human Trophoblast[1]

Miao Chang[3,4], **Debashree Mukherjea**[3,4], **Ryan M. Gobble**[4], **Kathleen A. Groesch**[4], **Ronald J. Torry**[5], and **Donald S. Torry**[2,4]

4*Department of Medical Microbiology, Immunology and Cell Biology, and Department of Obstetrics and Gynecology, Southern Illinois University School of Medicine, Springfield, Illinois 62794*

5*College of Pharmacy and Health Sciences, Drake University, Des Moines, Iowa 50311*

Abstract

Placental growth factor (PGF, previously known as PlGF) is prominently expressed by trophoblasts in human placenta, whereas most nontrophoblast cells express low levels of *PGF* mRNA under normal physiological conditions. We have shown that hypoxia decreases *PGF* expression in the trophoblast, but little is known about transcriptional regulation of *PGF* gene expression. We sought to determine promoter regions of the human *PGF* gene that contribute to its restricted high constitutive expression in the trophoblast. Overlapping putative promoter regions of human *PGF* gene encompassing −1.5 kb were cloned into reporter vectors and co-transfected into trophoblast and nontrophoblast cell lines. Promoter activity generated by a −1.5-kb clone was significantly higher in trophoblasts than in nontrophoblasts. Selective deletion mutants showed that a clone encompassing the *PGF* (−828/+34) region generated promoter activity similar to the −1.5-kb region in the trophoblast. However, deletion of another 131 bp from this subclone (−698/+34) resulted in significantly less promoter activity in the trophoblast. The (−828/−698) region significantly enhanced activity of a minimal promoter construct in trophoblast but not in nontrophoblast cells, suggesting that this region contributes to regulating *PGF* transcription in the trophoblast. Site-directed mutagenesis of a glial cell missing 1 (GCM1) motif in the 131-bp region significantly decreased enhancer activity in the trophoblast. Furthermore, overexpression of GCM1 significantly increased PGF −1.5-kb promoter activity and *PGF* mRNA expression in trophoblast and nontrophoblast cells. Forced overexpression of GCM1 restored *PGF* expression in the hypoxic trophoblast. These data support a functional role for GCM1 contributing to constitutively high trophoblast *PGF* expression and is the first direct evidence of an oxygen-responsive, trophoblast-specific transcription factor contributing to the regulation of *PGF* expression.

Keywords

gene regulation; growth factors; oxygen tension; placenta; placenta growth factor; placental growth factor; PlGF; pregnancy; transcription; trophoblast

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[2]Correspondence: Donald S. Torry, Department of Medical Microbiology, Immunology and Cell Biology, Southern Illinois University School of Medicine, P.O. Box 19626, Springfield, IL 62794-9626. FAX: 217 545 3227; e-mail: dtorry@siumed.edu.
[3]These authors contributed equally to this work.

Introduction

Placental growth factor protein (PGF, previously known as PlGF) is a member of the vascular endothelial growth factor (VEGF) family of proangiogenic factors [1] and is highly expressed by trophoblasts [2,3] and decidual natural killer cells [4] within the human maternal-fetal interface. PGF exists as different protein isoforms due to alternative splicing of the mRNA from a single copy gene [5,6], and four isoforms are expressed by trophoblasts [7]. PGF is chemotactic, mitogenic, and angiogenic for cultured endothelial cells and induces angiogenesis in vivo [8]. PGF overexpression produces abundant angiogenesis in normal mouse tissues [9] and stimulates collateral vessel growth in ischemic tissues [10]. Although PGF expression is normally low in most other organs/tissues, expression is inducible and required in tissues undergoing pathological angiogenesis [11], including vascularized tumors [12]. In addition to its known angiogenic activity, PGF promotes extravillous trophoblast proliferation [13], while data from our laboratory [14] and others [15] have shown that PGF can also function as a survival factor for trophoblasts. Furthermore, PGF induces relaxation of human placental vessels, which likely contributes to the reduction in blood flow impedance within the fetoplacental circulation [16]. Thus, PGF may contribute to trophoblast function as well as vascular development and function in the placenta.

We have shown that serum titers of PGF protein increase dramatically early in the second trimester of normal human pregnancy, whereas titers are significantly reduced in women diagnosed with preeclampsia [17]. Subsequently, it was found that decreased blood [18,19] and urinary [20] levels of PGF protein early in pregnancy are promising predictors of the subsequent development of preeclampsia. Quantitative studies have confirmed that preeclamptic trophoblasts express less *PGF* mRNA and protein than normal trophoblasts [21,22], and reduced bioavailability of PGF and VEGF produces a preeclampsia-like syndrome including hypertension, proteinuria, and glomerular endotheliosis in a pregnant rodent model [23]. Aberrations in trophoblast and/or leukocyte PGF production may compromise endothelial cell, trophoblast, and vascular function during gestation and contribute to the pathophysiologies commonly noted in perfusion-compromised pregnancies [24]. Although low oxygen tensions are known to decrease *PGF* expression in trophoblasts [3,21], the intrinsic molecular and cellular mechanisms that function to regulate *PGF* gene expression in human trophoblasts are not known. In this study, we describe for the first time the unique functional transcriptional regulatory regions of the human *PGF* gene and demonstrate that the transcription factor glial cell missing 1 (GCM1) contributes to the constitutive expression of *PGF* in the human trophoblast under different oxygen tensions.

Materials and Methods

Computational Analysis of the *PGF* 5′UTR for Putative Promoter Regions

As a reference to begin to determine the functional promoter region of human *PGF*, a 2500-bp upstream sequence of the human *PGF* gene was analyzed using two independent search engines: WWW Promoter Scan from the National Institutes of Health (Bethesda, MD; http://www-bimas.cit.nih.gov/molbio/proscan/) [25] and Genomatix PromoterInspector (Munich, Germany; http://genomatix.de). These promoter scan programs are designed to locate regions of DNA that contain significant numbers and types of known transcription factor binding sites associated with eukaryotic polymerase II promoter sequences.

Cloning of 5′ *PGF* UTR

Human yeast artificial chromosome hYAC 964_e_2 was used as the template for generating *PGF* upstream regions. Different lengths of the 5′ untranslated upstream region of *PGF* were generated by PCR amplification using primer sequences given in Table 1. The PCR cycling

conditions were: 95°C for 3 min, 30 cycles of 95°C for 1 min, 63°C for 1 min, and 70°C for 2 min. Resultant PCR amplicons of the appropriate predicted sizes were cloned into the beta-galactosidase reporter vector pBlue-TOPO-TA (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions and transformed into chemically competent DH5-α *E.coli* cells, and plasmid DNA was isolated by QIAprep Miniprep kit (Qiagen, Valencia, CA). All clones were sequenced in both directions (University of Illinois Biotechnology Center, Urbana, IL), and the validity of the sequences was verified against the published human genome sequence.

Cell Culture

Human choriocarcinoma cell lines JEG-3 and JAR and nontrophoblast cell lines hEK-293 (embryonic kidney; all from ATCC, Manassas, VA), HeLa (cervical carcinoma, gift from Dr. Mary McAsey, Southern Illinois University School of Medicine), and MCF-7 (breast carcinoma, gift from Dr. Jay Wimalasena, University of Tennessee) were grown in Dulbecco modified Eagle medium (DMEM; Fisher Scientific, Hanover Park, IL) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO) and antibiotics (penicillin G [50 U/ml] and streptomycin [50 μg/ml]; Fisher Scientific). Cells were cultured under 21% O_2 or 1% O_2 oxygen tensions as previously described [22].

Transient Transfection Assays

Transfections of the cell lines were performed using ExGen-500 (MBI Fermentas, Hanover, MD) according to the manufacturer's instructions. In experiments testing *PGF* promoter responses, 1.5 μg of the various *PGF* reporter constructs were co-transfected with 0.5 μg of pRSVLuc vector, and cells were incubated at 37°C. For GCM1 overexpression experiments, 0.75 μg of *PGF* −1.5-kb clone was cotransfected with either 1.0 μg of pHA-GCMa [26] (gift from Dr. Hungwen Chen, Institute of Biological Chemistry, Academia Sinica, Taiwan) or 1.0 μg of its corresponding empty vector pEF1/MycHis. Transfection efficiencies in these experiments were controlled with 0.25 μg of pRSVLuc. For GCM1 overexpression under hypoxic conditions, JEG-3 and JAR cells were transfected with 2 μg of pHA-GCMa or pEF1-HisMyc and cultured under 21% O_2 or 1% O_2 conditions for 24 h. For all experiments, the transfection medium was changed and fresh medium (DMEM + 10% fetal calf serum) was added 3 h after transfection. After an additional 48 h, cells were lysed and chemiluminescence detected using the Dual Light Kit (Applied Biosystems, Foster City, CA). All transfections were done in duplicate, and each individual lysate was tested in duplicate. Results were quantified using a Beckman Coulter LD400 Luminometer with dual injectors (Fullerton, CA). Luciferase values were used to normalize beta-galactosidase values to control for transfection efficiency. Luciferase and beta-galactosidase activities expressed by untransfected cell lysates were used as background and subtracted from all transfected cells. Luciferase readings were averaged across a replicate group, and each luciferase reading was corrected by dividing the group average luciferase reading by the individual luciferase reading. This corrected luciferase reading was then multiplied to the average beta-galactosidase reading to give a corrected beta-galactosidase activity. Relative changes in expression levels were calculated as fold differences in beta-galactosidase activity from a control clone in which the region from −1521 to −650 was inserted in an opposite orientation (−1521/−650opp). This control clone produced no significant beta-galactosidase activity in any of the cell lines ($P = 0.19$, paired Student *t*-test versus nontransfected cells). Therefore, basal beta-galactosidase values of the control (−1521/−650opp) clone were set to equal 1 to calculate relative fold changes in promoter activity for each transfection. Statistical analyses were performed between groups with the Student *t*-test, and significance was set as $P < 0.05$. All experiments were repeated at least three times, and all data is presented as mean ± SEM.

Functional Analysis of the *PGF* (−828/−698) Region

The clone encompassing (−1521/+34) was used as the template for PCR amplification of the (−828/−698) region with forward and reverse primers (5′-GAATTCGTCCATTCGACATATGCAGG-3′ and 5′-GAATTCTAACCGCCTCTGCAGGAG-3′, respectively). PCR reactions were carried out using puReTaq Ready-To-Go PCR Beads (Amersham Biosciences, Piscataway, NJ), and the cycling conditions were 94°C for 5 min followed by 31 cycles of 94°C for 30 sec, 56.6°C for 30 sec, and 72°C for 30 sec. An additional 10 min at 72°C was included at the end of 31 cycles. PCR amplicons were analyzed on 1.0% agarose gels to verify expected length, cloned into the pBlue-TOPO-TA, and subsequently subcloned into pMLuc-2 (Novagen, San Diego, CA). Clones were identified by bidirectional sequencing to verify sequence and orientation of the insets. Clones with sense or antisense orientation of (−828/−698) region were named pE173-MlucS and pE173-MlucAS, respectively. Transient transfection assays were performed as above except that chemiluminescence was detected with Dual-Luciferase Reporter Assay system (Promega, Madison, WI). Relative changes in expression levels were calculated as fold differences in corrected Renilla luciferase activity from empty pMLuc-2 vector.

Site-Directed Mutagenesis

GCM1 site mutation (indicated by lowercase nucleotides) was introduced with mutagenic primers (GCM-MS 5′-CCGAGAGCACCCCTACCTtgATATGTCGAATGGAC-3′ and GCM-MAS 5′-GTCCATTCAACATATcaAGGCAGGGGTGCTCTCAA-3′) using the (−844/−678) clone pE173-MlucS as template. These mutagenic primers were extended during PCR cycling with Pfu Turbo DNA polymerase (Quik Change XL Site-Directed Mutagenesis kit; Stratagene, LaJolla, CA). Dpn1 digestion was carried out afterwards to digest the potential DNA template and to select for the mutants. Mutants were confirmed by bidirectional sequencing and verified against pE173-MlucS and named pE173-GCM. Transient transfection assays were performed as above. GCM1 function was determined as fold differences in Renilla luciferase activity between pE173-GCM and pE173-MlucS.

Real-Time RT-PCR

Cells were lysed 48 h after transfection. RNA was extracted and 200 ng of total RNA converted to cDNA with iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA), and 1/10th of the cDNA was subjected to quantitative PCR using primers: (F): 5′-AGAAGATGCCGGTCATGAG-3′ and (R): 5′-ACACTTCCTGGAAGGGTAC-3′. Ribosomal protein (*RPL32*) was used as the normalization control gene and was amplified with *RPL32*(F): 5′-CCCAAGATCGTCAAAAAGA-3′ and *RPL32*(R): 5′-TCAATGCCTCTGGGTTT-3′ [27]. PCR reactions were performed in the iQ5 Real Time PCR Detection System with iQ SYBR Green Supermix (Bio-Rad). The cycling conditions for *PGF* and *RPL32* were: 95°C for 4.5 min, 40 cycles of 95°C for 30 sec, 62°C (*PGF*) or 53°C (*RPL32*) for 30 sec, and 72°C for 30 sec. PCR reaction efficiencies were monitored and found to be 93.7% (*PGF*) and 95.1% (*RPL32*). *PGF* expression was normalized to *RPL32*, and relative change of expression between treatment and control was calculated by the $2^{-\Delta\Delta CT}$ formula [28].

Immunoblots for HA-GCM1 Overexpression

Cells were lysed 48 h after transfection in lysis buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 10 mM PMSF, 1 mM Na_3VO_4, and 10 nM NaF) supplemented with complete protease inhibitor cocktail (Calbiochem, San Diego, CA), and protein concentrations were determined using the DC protein Assay kit (Bio-Rad). Ten micrograms of each cell lysate was separated on 12% SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked with 5% BSA in Tris-buffered saline containing 0.1 % Tween-20 (TBST) for 1 h at room temperature and incubated with rabbit anti-HA antibody (1:2000; Bethyl,

Montgomery, TX) overnight at 4°C. Membranes were washed with TBST 3 times, incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:10 000; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature, then washed 3 times with TBST and once with TBS. Immunoreactivty was detected by chemiluminesence as described (Amersham Biosciences) for 1 min. Membranes were stripped (0.2 M glycine [pH 2.2], 0.1% SDS, and 10% Tween 20) for 20 min at room temperature, then reprobed for human β-actin (Sigma) to control for loading consistency.

GCM1 Binding Assay

GCM1 binding to the potential binding site contained within the *PGF* promoter was performed as described [29] with minor modifications. Briefly, equimolar concentrations (10 pmol) of a biotinylated 42-mer oligonucleotide containing triplicate repeats of the putative GCM1 sequence (5′*ATGCAGGC*3′) present in the −828/−698 region of *PGF* (5′ CAT*ATGCAGGC*AGGCAT*ATGCAGGC*AGGCATATGCAT*ATGCAGGC*AGG 3′) or a non-sense control sequence (5′ AAGCTTTAAATTCCGCCCAAGTTTTAAATTCCG CCCAAGCTT 3′) were annealed. Each biotinylated double-stranded oligonucleotide was bound to avidin beads (VECTREX Avidin-D matrix; Vector Laboratories, Burlingame, CA), washed with 1 ml of TBST, and preincubated with 20 μg of nuclear extract from hEK-293 cells to reduce non-specific binding. Both the GCM1-nucleotide-bead matrix and the control nucleotide-bead matrix (24 μl) were combined with 20 μg of nuclear extract from hEK-293 cells, previously transfected with pHA-GCMa, in binding buffer (100 mM Tris [pH 7.5], 50 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 5.5 mM dithiothreitol, 10% Glycerol, 0.03% NP-40, 200 mM Na_3VO_4, 100 mM PMSF) containing 0.1 μg of sheared salmon sperm DNA and allowed to incubate for 30 min with end-over-end rotation at room temperature. The matrix was pelleted by brief centrifugation, unbound proteins in the supernatant were removed, and the beads were washed twice with 1 ml of binding buffer. The beads were resuspended in 30 μl of 4×Laemmli sample buffer (Sigma), boiled for 5 min, and centrifuged to pellet the matrix. Proteins in the supernatant were separated on a 10% SDS-PAGE gel, transferred to nitrocellulose membrane, and immunoblotted for HA using conditions described above.

Results

Generation of *PGF* Reporter Constructs

To determine functional promoter regions responsible for high constitutive trophoblast-specific expression and to identify regions that may regulate trophoblast restricted transcription of *PGF*, selective reporter clones of the *PGF* 5′ UTR were generated (Fig. 1). Two independent promoter analysis programs predicted putative, overlapping, basal promoter regions between −538 bp and −346 bp and −666 bp and −416 bp upstream of the first ATG codon. These predicted promoter sequences overlap a common 122 bases and lie within 20–96 bp, respectively, of the single transcription start site of the h*PGF* gene (−320 bp) [1,30]. Three clones—PGF (−305/+34), PGF (−698/+34), and PGF (−828/+34)—contain the first ATG codon of the translation start site of h*PGF* and extend to −828 bp upstream. Clone PGF (−698/−284) contains the predicted promoter sites from the −346 bp to −666 bp region. Clones PGF (−1042/−650) and PGF (−1521/−650) contain regions upstream of the predicted promoter sites. Clone PGF (−1521/+34) spans 1.5 kb upstream of the *PGF* gene.

Functional Analysis of h*PGF* Promoter Constructs in Trophoblast Cell Lines

The promoter constructs were transfected in two choriocarcinoma cell lines, JEG-3 and JAR, to determine functional activities. Both of these trophoblast cell lines have been shown to express high levels of *PGF* mRNA under standard culture conditions [3,25]. The three clones that expressed significant promoter activity were: −1.5-kb clone (−1521/+34), (−828/+34), and (−698/+34) (Fig. 2A). In both trophoblast cell lines, promoter activity of the −1.5-kb clone

(1521/+34) was significantly higher than that of cells transfected with the (−1521/−650opp) control clone. JEG-3 cells showed a 14.3 ± 1.1 ($P < 0.001$)-fold increase in beta-galactosidase activity, while JAR cells showed 10.5 ± 2.1 ($P < 0.005$)-fold increase in beta-galactosidase activity. There was no significant difference ($P = 0.63$) in the expression levels of the −1.5-kb clone between the two trophoblast cell lines. These results confirm the ability of the −1.5-kb region of the *PGF* 5′UTR to drive reporter gene expression in the trophoblast.

Trophoblast cells transfected with clone (−698/+34), which contains both the predicted promoter region as well as the gene proximal 284-bp region, produced significantly ($P < 0.05$) higher promoter activity in JEG-3 cells (5.2 ± 0.5-fold) and in JAR cells (4.3 ± 1.6-fold) as compared to promoter activity produced by the control clone. However, the promoter activity exhibited by this clone was significantly lower than promoter activity produced by the −1.5-kb clone in JEG-3 cells ($P < 0.001$) and in JAR cells ($P < 0.05$; Fig. 2A).

Interestingly, clone (−828/+34), which contains an additional 131 bp upstream of the (−698/+34) region, produced 10.5 ± 0.7–fold promoter activity in JEG-3 cells and 9.2 ± 3.3 ($P < 0.05$)-fold promoter activity in JAR cells. There was no significant difference ($P > 0.07$) observed in the promoter activity of clone (−828/+34) and that of clone (−1.5 kb/+34) in JEG-3 or JAR cells (Fig. 2A). This clone produced a 2-fold increase in promoter activity compared to the (−698/+34) clone, suggesting that the region between −828 bp and −698 bp contains elements that can augment high trophoblast-specific promoter activity.

Subsequent analyses of various deletion mutant clones of the −1.5-kb region showed that clones encompassing (−305/+34), (−698/−284), (−1042/−650), and (−1521/−650) produce little to no promoter activity in JEG-3 cells (Fig. 2B). Identical results were obtained with all of these deletion clones in JAR cells (data not shown). In particular, clone (−698/−284), which encompasses both of the predicted putative promoter regions, produced low promoter activity that was not significantly above control levels ($P = 0.7$) in either JEG-3 or JAR cells. Thus, the predicted promoter region between −698 bp and −284 bp is not sufficient alone to produce promoter activity in trophoblast cells and requires the involvement of accessory elements for maximal activity.

Functional Analyses of h*PGF* Promoter Constructs in Nontrophoblast Cell Lines

To determine regions in the *PGF* promoter responsible for directing tissue-specific expression, we compared the promoter activity of the clones in the two trophoblast cell lines with that generated in three different nontrophoblast cell lines: hEK-293, HeLa, and MCF-7 cells. Promoter activities produced by the −1.5-kb clone in hEK-293 (4.2 ± 1.6–fold) and MCF-7 cells (3.6 ± 1–fold) were significantly higher than compared to the control clone (−1521/−650) opp (Fig. 3A). Clone −1.5 kb failed to produce promoter activity above background in HeLa cells. Although activity in the hEK-293 and MCF-7 cells was significantly higher than control clone activity, this intermediate level of activity was significantly ($P < 0.05$) lower compared to the mean activity of this region in the trophoblast cells. To validate these reporter clone studies, we determined the relative expression levels of endogenous *PGF* mRNA in the cells by real time RT-PCR. Expression of *PGF* mRNA in the HeLa and hEK-293 cell lines under standard culture conditions was found to be much lower than that seen in the trophoblast cell lines (Fig. 3B). Endogenous *PGF* mRNA was almost undetectable in HeLa cells, which is in agreement with a previous report [31] and the promoter clone data above. Similarly, the intermediate activity of the 1.5-kb clone in MCF-7 and hEK-293 cells corresponds well to the relative levels of *PGF* mRNA found in hEK-293 cells and previously reported by us in MCF-7 cells [32]. Collectively, these findings show that the promoter activity produced by the −1.5-kb clone in trophoblast and nontrophoblast cells accurately reflects *PGF* mRNA expression levels by the different cell lines and further highlights cell type-specific regulation of *PGF* expression. Similar to that seen in trophoblast cells, undetectable levels of promoter activity

were produced by clones (−305/+34), (−698/−284), (−1042/−650), and (−1521/−650) in all three nontrophoblast cells (data not shown). The ability of the (−1.5 kb/+34) clone, but not clone (−305/+34), to produce significant activity in some, but not all, nontrophoblast cells suggests that regions between −1.5 and −305 may be important for regulating promoter activity in these cells in a similar manner to trophoblast cells.

Functional Analyses of Tissue-Specific Regulatory Regions of Human *PGF*

To further characterize potential tissue-specific regulatory regions of the *PGF* 5′ UTR, promoter activities of clones (−698/+34) and (−828/+34) were determined in nontrophoblast cells (Fig. 4). Clone (−698/+34) produced approximately 3-fold more promoter activity in HeLa and hEK-293 cells than the control clone (−1521/−650opp). In contrast, addition of the 131-bp sequences (clone −828/+34) produced approximately 6-fold less activity versus that produced by the (−698/+34) clone in the same cells. There was little difference in mean promoter activity of these clones in the MCF-7 breast cancer cells. Collectively, this pattern of promoter activity is opposite to that seen in trophoblast cells by these clones, where inclusion of the 131-bp region between −828 and −698 resulted in an approximate 2-fold increase in promoter activity (Fig. 4). Thus, the presence of this 131-bp region in clone (−828/+34) compared to clone (−698/+34) decreases promoter activity in most nontrophoblast cells, but increases promoter activity in trophoblast cells.

GCM1 Functions to Regulate *PGF* Promoter Activity and Gene Expression

To specifically confirm the functional ability of this 131-bp *PGF* promoter region to promote transcriptional activity in trophoblasts, the *PGF* region encompassing (−828/−698) was cloned downstream of a minimal thymidine kinase (TK)-driven Renilla luciferase cassette in both sense and antisense orientations. In trophoblasts, both the sense- and antisense-oriented 131-bp element generated a significant increase in basal TK promoter activity (Fig. 5A). In contrast, the 131-bp element in either orientation did not significantly influence TK promoter activity in the HeLa or hEK-293 cells. Differences in functional activity of these clones in the cells suggest the presence of trophoblast-specific transactivating factors that recognize unique DNA elements within the region.

To elucidate functional elements that may be responsible for enhancing *PGF* gene expression in the trophoblast, we analyzed the (−844/−678) region electronically and identified a motif at position −818 to −810 with 87.5% sequence homology with consensus GCM1 motif (A/G) CCCGCAT [33]. In vitro binding experiments were used to investigate the functional ability of this motif to bind GCM1 protein (Fig. 5B). These studies established that the putative GCM1 motif in *PGF* is capable of directly binding exogenously expressed GCM1 protein in nuclear extracts of transfected hEK-293 cells (Fig. 5B). These results are in agreement with previous findings showing that a sequence with a similar mismatch maintains high binding capacity for *Drosophila* and murine GCM1 [34]. GCM1 protein is expressed in trophoblast cells [35] and has been shown to specifically regulate expression of certain genes in these cells [36]. To verify that the putative GCM1 element functions to regulate *PGF* enhancer activity, we carried out site-directed mutagenesis of the GCM1 motif in the (−844/−678) region. Transient expression experiments of the promoter constructs were performed in trophoblast and nontrophoblast cells to compare functional activity of wild-type GCM1 to mutated GCM1 motif (Fig. 5C). Mutation of the GCM1 motif resulted in significantly less promoter activity compared to wild-type GCM1 in the trophoblast. However, there was no significant difference in activity between mutated and wild-type GCM1 constructs in nontrophoblast cells. These data provided the initial evidence suggesting that the GCM1 motif within the (−844/−678) region of *PGF* contributes to transcriptional activity in the trophoblast.

To confirm the role of GCM1 in regulating *PGF* transcriptional activity, we overexpressed exogenous GCM1 in trophoblast and nontrophoblast cells in the presence of the −1.5-kb reporter construct (Fig. 6A). Real time RT-PCR analyses indicated that relative expression levels of endogenous GCM1 mRNA were similar between the two trophoblast cell lines and that they expressed approximately 32-fold more GCM1 mRNA than did the two nontrophoblast cells (data not shown). GCM1 overexpression induced significant *PGF* promoter activity in JEG-3 (3.1-fold), JAR (11.0-fold), HeLa (3.4-fold), and hEK-293 (72-fold) cells (Fig. 6B). To confirm the ability of GCM1 to specifically induce *PGF* gene expression, we overexpressed GCM1 protein in HeLa and hEK-293 cells and monitored endogenous *PGF* mRNA expression (Fig. 6C). The significant increase in *PGF* promoter activity following overexpression of exogenous GCM1 in these cells (Fig. 6B) was reflected in a significant increase in expression of *PGF* mRNA in the cells (Fig. 6C). Overexpression of GCM1 increased *PGF* mRNA in HeLa cells, with the highest induction of ~108-fold with 1.0 μg of pHA-GCM1 as compared to transfections with backbone vector pEF1-MycHis. Similarly, in hEK-293 cells, as little as 0.5 μg pHA-GCM1 increased *PGF* mRNA expression 19-fold, and the induced *PGF* mRNA expression levels were similar up to 2.0 μg of pHA-GCM1. Transfection of the GCM1 expression vector into trophoblast cells also increased endogenous *PGF* mRNA levels ~30% in the already highly expressing JEG-3 cells and approximately 2.5- to 4.0-fold in the JAR cells (data not shown).

Low oxygen tension has been shown to specifically decrease *PGF* mRNA expression in trophoblasts [3,37]. We sought to determine if GCM1 mediates this effect since others have shown that low oxygen tension decreases *GCM1* mRNA expression in BeWo cells [38]. We performed qRT-PCR and confirmed that *GCM1* mRNA expression was significantly decreased (>80%) when JEG-3 cells were cultured in 1% O_2 (data not shown). To determine if maintenance of GCM1 expression could rescue decreased *PGF* mRNA expression in hypoxic trophoblasts, we overexpressed GCM1 in hypoxic JEG-3 cells (Fig. 6D). As we have previously shown, exposure of JEG-3 cells to 1% O_2 significantly decreased *PGF* expression by 24 h. However, overexpression of exogenous GCM1 in the hypoxic trophoblast increased endogenous *PGF* mRNA levels about 6-fold above those in hypoxic cells lacking GCM1 and about 3-fold above levels noted in normoxic cells. Similar results were observed in JAR trophoblast cells (data not shown).

Discussion

At the maternal-fetal interface, expression of *PGF* mRNA is normally high in trophoblasts [22] and uterine lymphocytes [39]. In contrast, most other cell types show little to no *PGF* expression under normal physiologic conditions [40]. The mechanisms underlying the differential high *PGF* expression in trophoblasts as compared to other cell types are not known. Our study focused on defining the regions of the *PGF* gene responsible for regulating its uniquely high and constitutive transcription in trophoblast cells. High *PGF* promoter activity was produced in two different trophoblast cell lines by a 1.5-kb clone that spans bases −1521 to +34. However, a significantly lower level of activity was observed when this clone was transfected in three different nontrophoblast cell lines. The promoter activity correlated directly with the endogenous levels of *PGF* mRNA reported in these cell lines by us [3] and others [6,31]. These data suggest that the −1521-bp region proximal to the *PGF* gene is capable of producing constitutive and trophoblast-specific promoter activity.

Transfection studies in both the trophoblast and nontrophoblast cell lines with various reporter gene constructs showed that the region between −828 bp and +34 bp of the *PGF* gene maintained a high level of activity in trophoblast cells and little activity in nontrophoblasts. We also determined that the region −698 bp to +34 bp can function as a basal promoter with reduced activity in trophoblasts and yet still be permissible for increased constitutive activity

in certain nontrophoblast cells. However, this region did not seem to significantly regulate the expression of *PGF* in the MCF-7 breast cancer line. This is in line with our previous findings showing that these cells express low but detectable levels of *PGF* mRNA [22].

We subsequently sought to determine what DNA elements might be present in the 131-bp region between −698 and −828 that mediate the increased transcriptional activity in trophoblasts. The 131-bp region between −828 bp and −698 bp significantly augmented minimal TK promoter activity independent of orientation in trophoblast, but not nontrophoblast, cells. These findings, which are suggestive of an enhancer-like effect, are similar to fold inductions noted for other placenta-specific enhancers [41,42]. In contrast, the same region did not show a significant effect on promoter activity in nontrophoblasts. Collectively, these results suggest the presence of trophoblast-specific elements within this 131-bp region.

A number of placenta- and trophoblast-specific elements have been identified that can regulate placenta and trophoblast expression of different genes. Placenta-specific elements include cAMP responsive elements, upstream regulatory element (URE), trophoblast specific element and URE1 of human gonadotropin α subunit hormone [31,33], chorionic somatomammotropin enhancer factor of the human chorionic somatomammotropin-B gene [34], and placental leptin elements (PLE)-1 and PLE-3 [30]. However, computer analyses of the 131-bp sequence between −698 bp and −828 bp and that of the entire 1.5-kb upstream region of the *PGF* gene did not reveal sequence homologies at 80% stringency for any of these sites.

Our in silico analyses did highlight several putative transcription factor binding elements within this 131-bp region, including Oct-1, SP-1, ETS, and transcription enhancing factor (TEF-1). Some of these putative elements have been shown to function at least in part to regulate transcription of various genes in the trophoblast [41,43–45]. However, most notable was the presence of a motif that shared high sequence homology (87.5%) with GCM1 binding sites [46]. Based on the trophoblast-specific expression of GCM1 [35], its ability to transcriptionally regulate other trophoblast-specific genes including syncytin [47] and aromatase [36], and its decreased expression in preeclampsia [48] and hypoxic trophoblasts [38], we focused on the corresponding DNA element for GCM1 as a regulator for *PGF* gene expression. This putative GCM1 element in human *PGF* specifically bound GCM1 proteins in direct binding assays, confirming its functional protein-binding capacity. Site-directed mutagenesis of the single GCM1 binding motif in the 131-bp region significantly inhibited the enhancing effect of this region in the trophoblast. However, the mutated GCM1 motif had no effect on the basal promoter activity of the reporter clone in nontrophoblast cells. To confirm the ability of GCM1 to regulate *PGF* promoter activity, we overexpressed GCM1 protein in both trophoblast and nontrophoblast cells. GCM1 overexpression increased activity of the full-length *PGF* 5′UTR (−1.5-kb region) in both cell types, but to differing degrees. Trophoblasts express high levels of *GCM1* (this report and [35]), whereas the nontrophoblast cells we tested (HeLa, hEK-293) express almost undetectable levels of *GCM1* mRNA. Forced overexpression of GCM1 significantly increased transcriptional activity of the −1.5-kb *PGF* reporter clone and resulted in significant increases (20- to 100-fold) in endogenous *PGF* mRNA expression in the nontrophoblast. In addition, increased expression of GCM1 increased endogenous *PGF* mRNA expression in the trophoblast. These data indicate that GCM1 is able to regulate *PGF* promoter activity and that GCM1 cell type-specific expression might be partially responsible for high *PGF* promoter activity in trophoblasts, but lower activity in most nontrophoblasts. Whether the other identified putative transcription factor binding elements contribute to trophoblast-specific *PGF* expression is currently under investigation.

Our data supports that GCM1 alone can positively regulate *PGF* promoter activity by direct interaction with *PGF* DNA; however, it is possible that other transcription factors may

cooperate with GCM1 to regulate its functional activity in a cell type-specific manner. For instance, coexpression of GCM1 and PITX1 or PITX2 synergistically increased transcriptional activity of a synthetic GCM1 reporter construct in trophoblast (JEG-3 cells), but repressed GCM1 activity in hEK-293 cells [49]. Similarly, functional activity of GCM1 in neuronal cell types has been shown to be negatively regulated by repressor proteins [50].

The ability of GCM1 to regulate *PGF* gene activity correlates well with their mutual expression patterns in trophoblasts in vitro and in normal and preeclamptic placentae. Sustained GCM1 expression is restricted to cytotrophoblasts and syncytiotrophoblasts in human placentae [35, 48,51]. Furthermore, expression of *GCM1* mRNA and protein is significantly lower in trophoblasts of late third trimester placentae than in midgestation trophoblasts [48]. Expression of PGF protein is temporally similar in that maternal systemic levels steadily decrease from a peak at approximately 26–30 wk gestation to term [17], and placental *PGF* mRNA expression is lower at term than in midgestation [22]. Placentae from near-term preeclamptic pregnancies express less GCM1 protein and mRNA and there are fewer GCM1 expressing trophoblast cells than are evident in gestational-age matched normal placentae [48]. These findings correlate well with the decreased PGF protein [17–20,52,53] and mRNA [22] expression noted in preeclamptic pregnancies. Furthermore, expression of GCM1 is significantly decreased in choriocarcinoma cells and primary term trophoblasts exposed to low oxygen tension [38]. Low oxygen tension is known to specifically decrease *PGF* expression in trophoblasts [3,37], which is mediated at least in part by decreased transcription (R.M. Gobble, D.S. Torry, et al. unpublished results). We confirmed that low oxygen tension decreased *GCM1* and *PGF* mRNA expression in trophoblast cells. Importantly, forced overexpression of GCM1 in hypoxic trophoblasts maintained high levels of *PGF* mRNA expression in the cells. Collectively, these results suggest that GCM1 plays a critical role in regulating *PGF* transcription in trophoblasts and that decreased trophoblast GCM1 expression noted in preeclampsia may contribute to the decreased expression levels of *PGF*.

It is increasingly evident that adequate growth and maintenance of the vasculature is an important component of successful placentation [54]. Furthermore, discrete threshold differences in growth factor expression may provide abnormal vascular growth and placentation [55]. Clearly, studies designed to determine tissue and cell type-specific regulatory regions of angiogenic growth factors like PGF in the placenta are needed. In summary, our studies have localized promoter regions contributing to constitutive expression of the *PGF* gene in human trophoblast. We have identified a 131-bp region located between −828 bp and −698 bp of the *PGF* gene that contributes to increased promoter activity in trophoblast cells and have identified GCM1 as a mediator of this enhancing effect. Furthermore, oxygen regulation of *PGF* gene expression in trophoblasts is reflected, at least in part, by the effects of oxygen on this trophoblast-specific transcription factor. Given the important role of PGF in regulating pathological angiogenesis [11], our identification of the putative transcription factor binding sites of the *PGF* gene may also highlight molecular mechanisms responsible for the increased regulation of PGF expression in well-vascularized tumors and wound healing.

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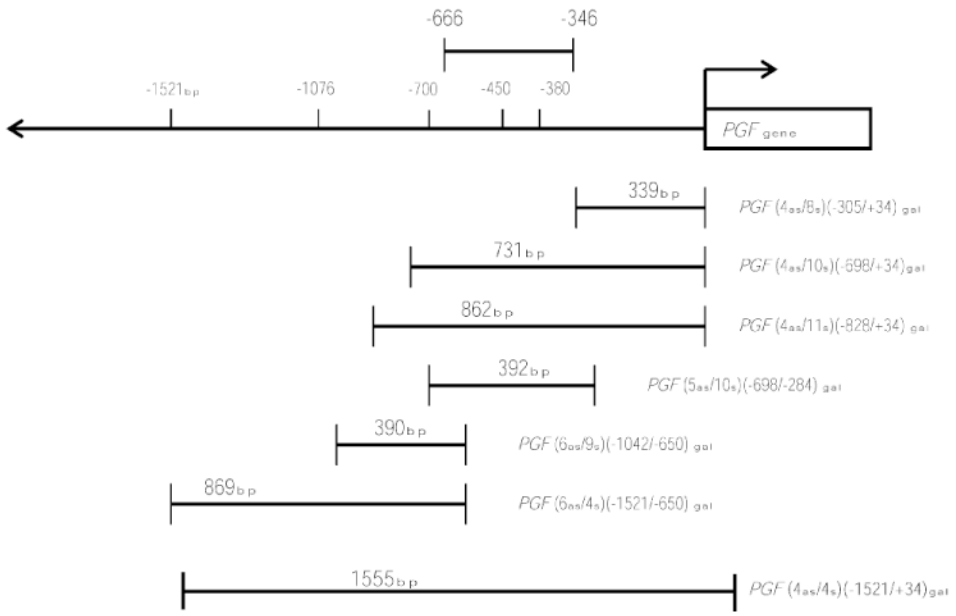

Fig. 1.

Schematic representation of *PGF* promoter clones. Putative *PGF* promoter clones were generated by ligating upstream regions of human *PGF* gene amplicons into a beta-galactosidase reporter vector. Clones of different sizes spanning a 1.5-kb upstream region of the *PGF* gene are depicted. Computational analyses of the upstream DNA sequences by two independent programs predicted that regions between −666 and −346 contained DNA binding motifs able to provide promoter activity.

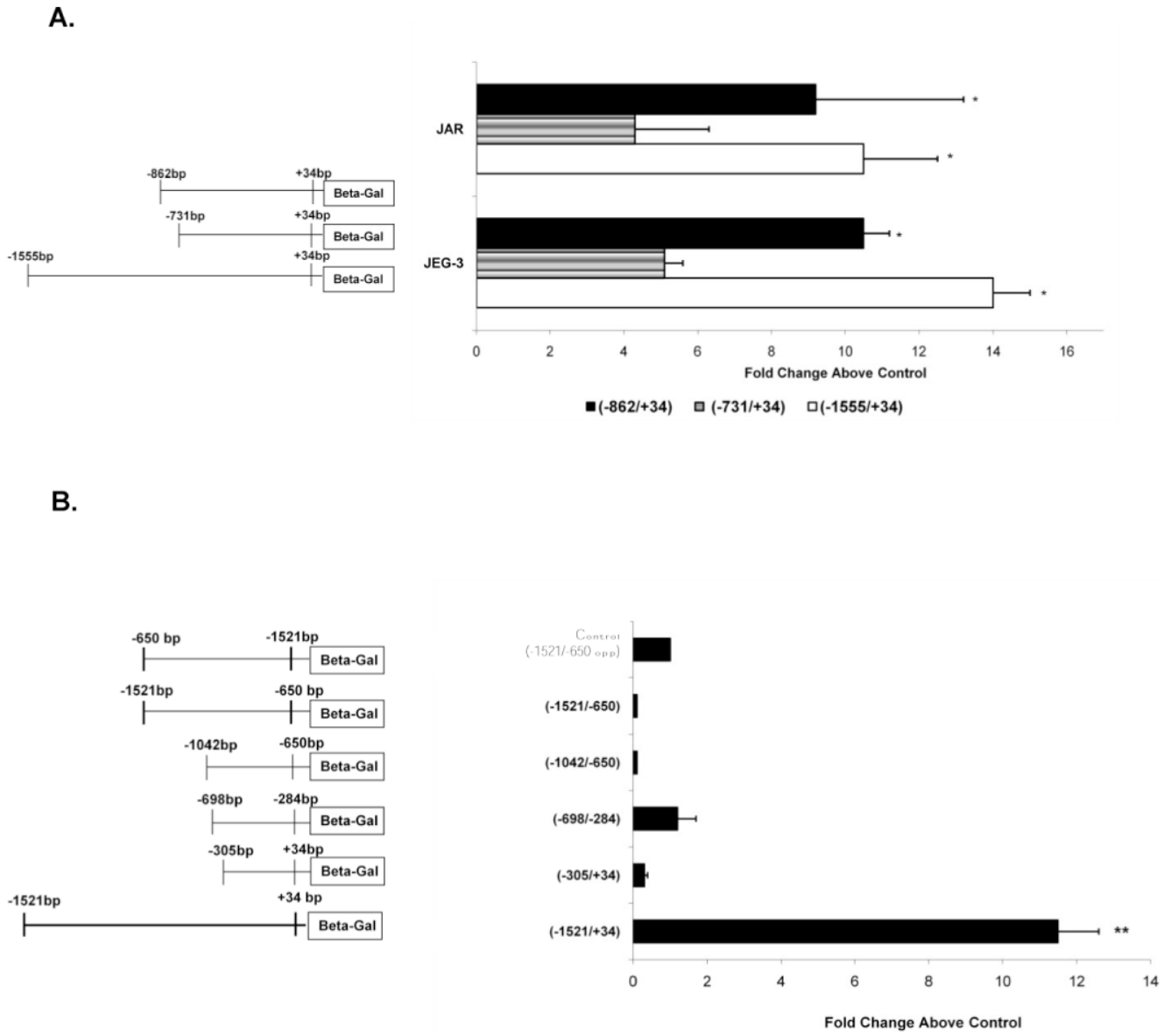

Fig. 2.

Functional promoter activity within 1.5 kb upstream of the *PGF* gene. **A**) JEG-3 and JAR cells were transfected individually with 1.5 μg of clone (−828/+34), clone (−698/+34), or the −1.5-kb clone (−1521/+34) and 0.5 μg of pRSVLuc. After 48 h, cell lysates were prepared and chemiluminesence measured as described in *Materials and Methods*. The relative fold increase in activity above levels produced by the transfection control clone (−1521/−650opp) for each of the clones in JEG-3 and JAR cells are shown. All activities shown are significantly higher than those produced by the control clone (set = 1). *, $P < 0.05$ versus activity produced by the (−698/+34) clone. **B**) Promoter activity produced by clones (−1521/+34), (−305/+34), (−698/−284), (−1042/−650), and (−1521/−650) in JEG-3 cells. **, $P < 0.05$ between activities of the promoter clones versus transfection control clone (−1521/−650opp) (set = 1).

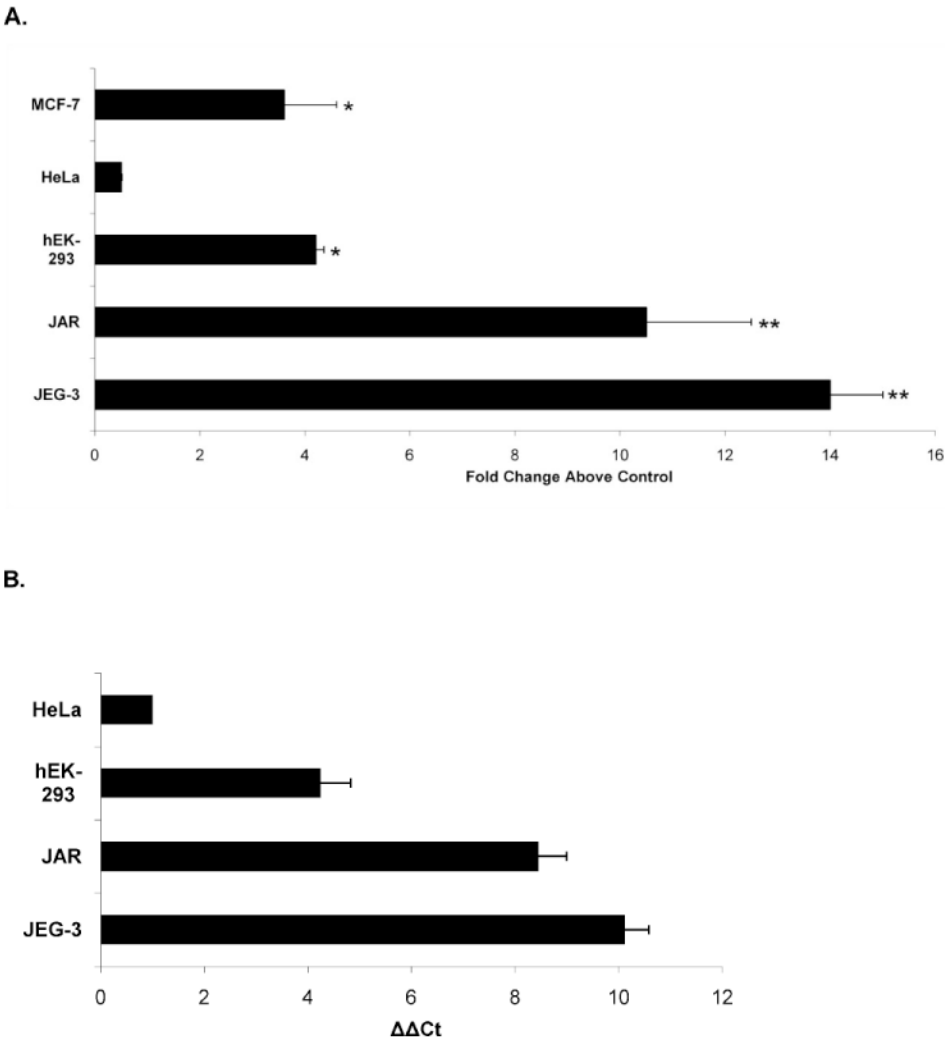

Fig. 3.

PGF promoter activity and mRNA expression are significantly lower in nontrophoblast cells than trophoblast cells. **A**) MCF-7, HeLa, hEK-293, JEG-3, and JAR cells were transfected with 1.5 μg of clone (−1521/+34) or 1.5 μg of control plasmid (−1521/−650opp) and 0.5 μg of pRSVLuc. Cells were processed and relative fold increase in activity above levels produced by the transfection control clone (−1521/−650opp) (set to = 1) in each cell type are shown. *, $P < 0.05$ comparing promoter activities produced by the −1.5-kb clone and (−1521/−650opp) control clone in each cell type. **, $P < 0.005$ comparison of promoter activity of the −1.5-kb clone in trophoblast cells to that produced in each nontrophoblast cell type. **B**) Relative *PGF* mRNA expression in trophoblast and nontrophoblast cell lines. Total RNA (200 ng) from each cell line was reverse transcribed into cDNA and subjected to qPCR for both *PGF* and *RPL32* detection. ΔCT of HeLa (CT^{Pgf}-CT^{RPL32})-1 is used as the normalization control, and ΔΔCT of each cell type was plotted to show *PGF* mRNA expression level relative to that in HeLa cells. Data represents mean values (± SEM) of at least three independent measurements.

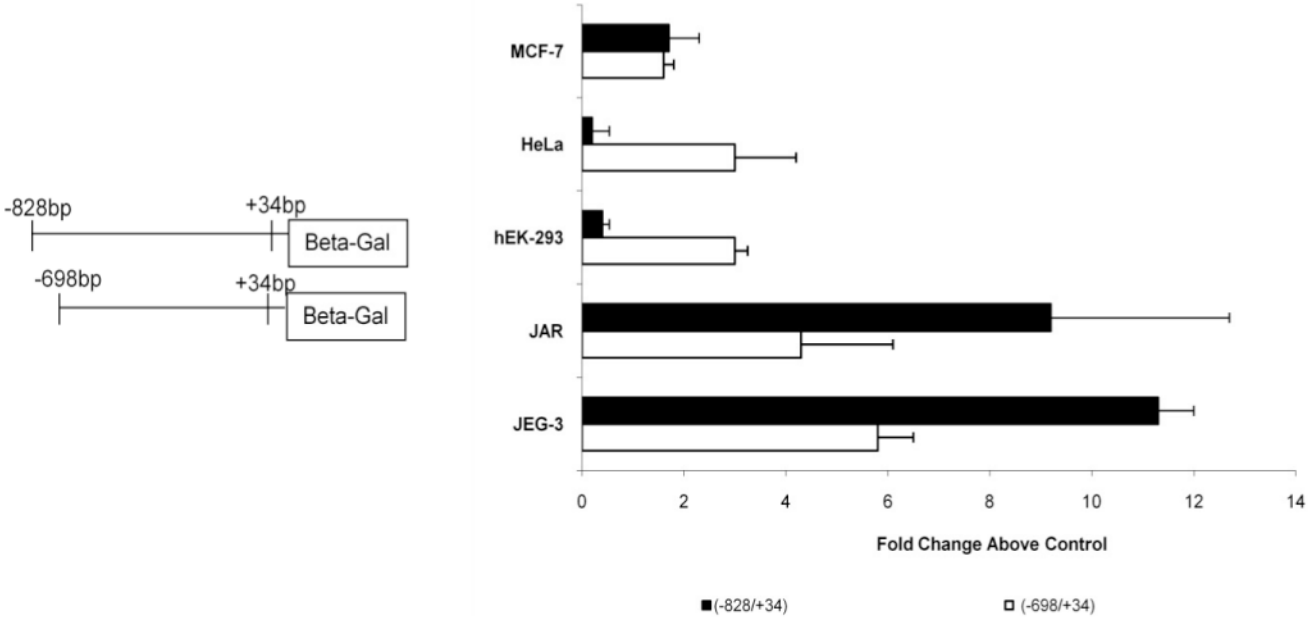

Fig. 4.

Tissue-specific *PGF* expression is mediated by the region between −828 and −698 bp upstream of the *PGF* translation start site. JEG-3, JAR, hEK-293, HeLa, and MCF-7 cells were transfected with 1.5 μg of clone (−698/+34) or clone (−828/+34) and 0.5 μg of pRSVLuc. Cells were processed and fold activity above background plotted as in Figure 2. Clone (−828/+34) produced high promoter activity in trophoblast cells JEG-3 and JAR, but not in the nontrophoblast cells hEK-293 and HeLa. Clone (−698/+34) produced relatively similar activity in the various cell lines.

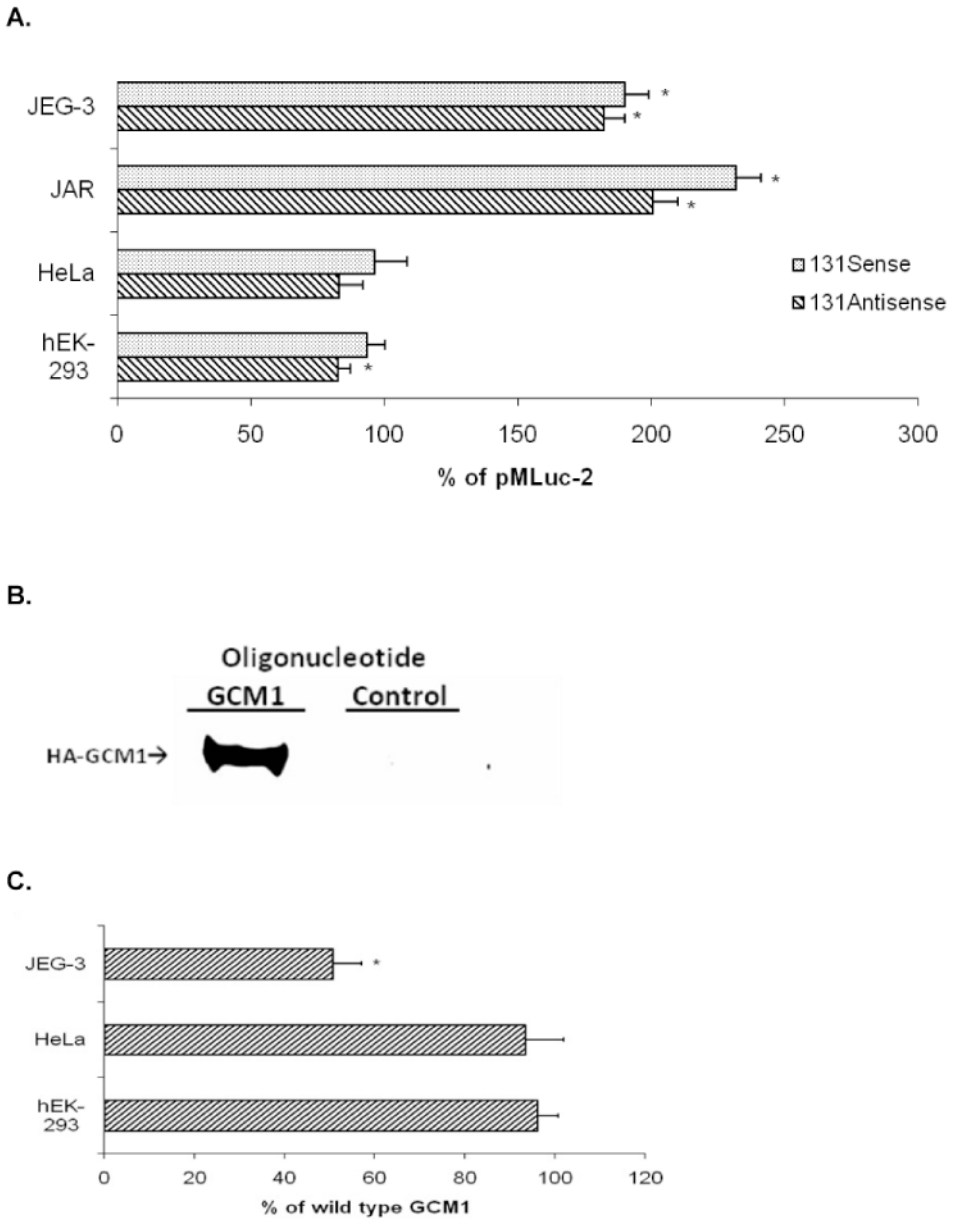

Fig. 5.

PGF (−828/−698) region enhances basal promoter activity in trophoblast but not nontrophoblast cells. **A**) JEG-3, JAR, HeLa, and hEK-293 cells were transfected with 1.5 μg of pE173-MlucS or pE173-MlucAS clones and 0.5 μg of pRSVLuc. Statistical analyses compared promoter activity of each individual clone with its parental control clone pMLuc-2 (set to 100%) in each cell type (*, $P < 0.005$). **B**) Nuclear extracts from hEK-293 cells previously transfected with pHA-GCM1 were incubated with bead-conjugated oligonucleotides containing either three copies of the putative GCM1 motif from human *PGF* (*GCM1*) or corresponding scrambled sequences (control). Western blots for HA-tagged GCM1 (HA-GCM1) were carried out to examine the binding ability of the GCM1 motif. **C**) GCM1 element of *PGF* (−828/−698) region in pE173-MLucS was mutated and transfected into JEG-3, HeLa, and hEK-293 cells. Activity of mutant GCM1 element relative to wild-type activity in each cell type was plotted (*, $P < 0.005$).

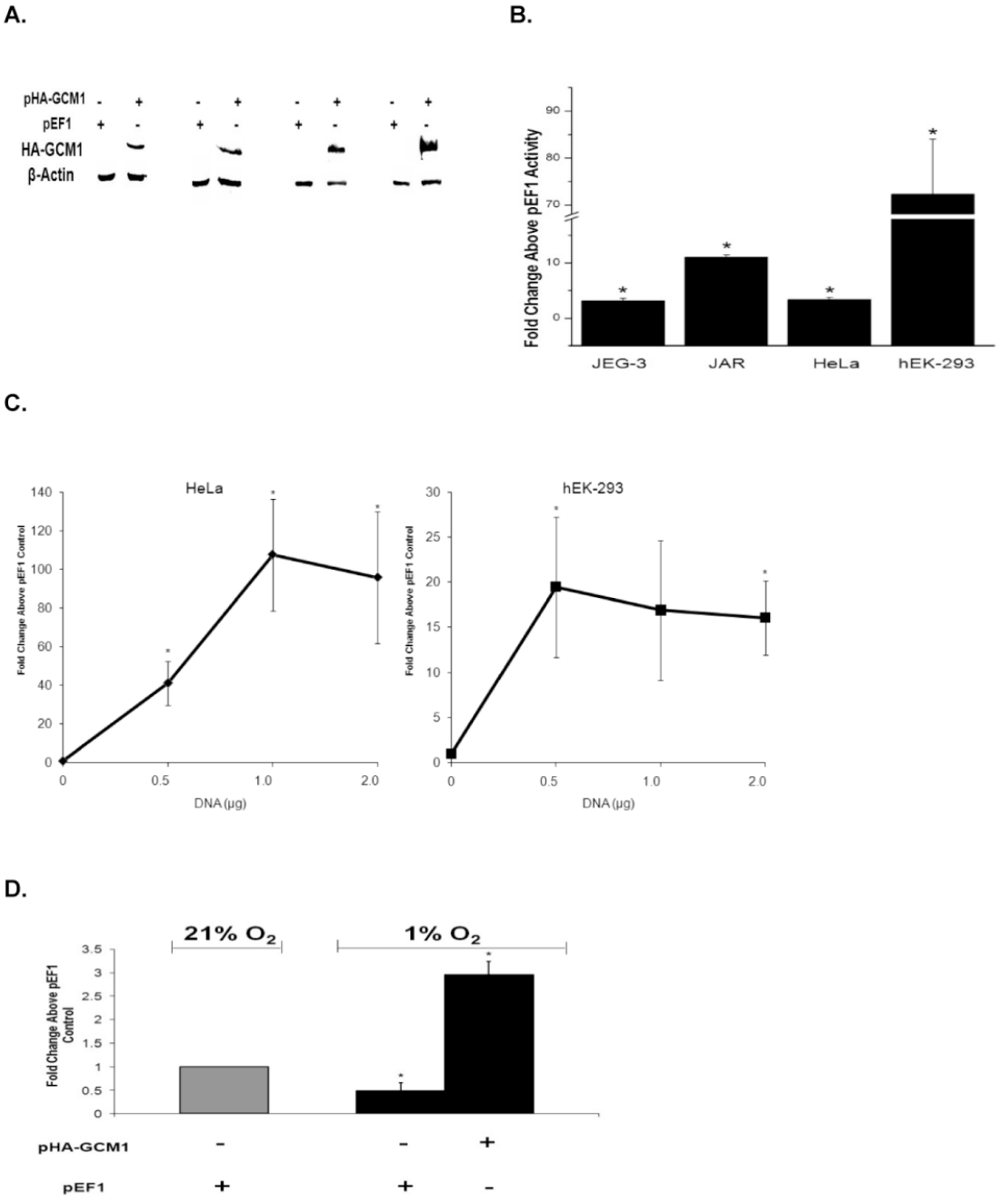

Fig. 6.

GCM1 regulates *PGF* promoter activity and mRNA expression. **A**) Expression of exogenous GCM1 was confirmed by Western blot for HA-tagged GCM1. Cells were transfected with pHA-GCM1 or a control empty vector (pEF1/MycHis) and immunoblotted for anti-HA and β-actin. Paired lanes correspond to JEG-3, JAR, HeLa, and hEK-293, respectively. **B**) GCM1 increased transcriptional activity of the −1.5-kb clone in both trophoblasts and nontrophoblasts. Cells were cotransfected with pHA-GCM1 or pEF1/MycHis and (−1521/+34) *PGF* reporter construct. *PGF* reporter activity was determined 48 h later and fold activity above cells transfected with pEF1/MycHis for each cell type plotted. GCM1 overexpression significantly increased PlGF −1.5-kb promoter activity in both trophoblast and nontrophoblast cells (*, $P <$

0.05). **C**) GCM1 overexpression increased (*, $P < 0.05$) *PGF* mRNA expression in nontrophoblasts. GCM1 overexpression was accomplished with increasing concentrations of pHA-GCM1, and qRT-PCR was performed 48 h later to determine *PGF* and *RPL32* mRNA expression. *PGF* mRNA expression was normalized to *RPL32* expression, and relative changes in *PGF* mRNA expression were compared to control transfections with the pEF1/MycHis empty vector (set = 1). **D**) GCM1 overexpression restores *PGF* mRNA expression in hypoxic trophoblasts. JEG-3 cells were transfected with GCM1 overexpression plasmid (pHA-GCM1) or pEF1/MycHis and cultured under either 21% O_2 or 1% O_2 for 24 h. Quantitative RT-PCR was performed to determine *PGF* and *RPL32* mRNA expression. *PGF* mRNA expression was normalized to *RPL32* expression and relative changes in *PGF* mRNA expression were compared to the result of pEF1/MycHis transfected cells cultured at 21% O_2 (set = 1). *, $P < 0.05$.

Table 1

Primer sequences used to generate the different human *PGF* promoter clones.

Primer name (sequence location)	Sequence
4 Antisense (+34 bp)	5′-GCAGGAAGCAAGGGAACAGCC-3′
8 Sense (−305 bp)	5′-AGCTACGGGAGGACCTGGAGT-3′
10 Sense (−698 bp)	5′-CCTGCAGAGGCGGTTAGATCA-3′
11 Sense (−828 bp)	5′-CCATTCGACATATGCAGGCAG-3′
5 Antisense (−284 bp)	5′-ACTCCAGGTCCTCCCGTAGCT-3′
6 Antisense (−650 bp)	5′-GTCCAGCGTCAGCCGTCAAGG-3′
9 Sense (−1042 bp)	5′-CTTCCCAAGGAGGCAACAGGC-3′
4 Sense (−1521 bp)	5′-CAGAAGGCAGTGAGAGGAGTGG-3′