Decreased PGF may contribute to trophoblast dysfunction in fetal growth restriction

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Abstract

Fetal growth restriction (FGR) threatens perinatal health and is correlated with increased incidence of fetal original adult diseases. Most cases of FGR were idiopathic, which were supposed to be associated with placental abnormality. Decreased circulating placental growth factor (PGF) was recognized as an indication of placental deficiency in FGR. In this study, the epigenetic regulation of PGF in FGR placentas and the involvement of PGF in modulation of trophoblast activity were investigated. The expression level of PGF in placental tissues was determined by RT-qPCR, immunohistochemistry and ELISA. DNA methylation profile of PGF gene was analyzed by bisulfite sequencing. Trophoblastic cell lines were treated with ZM-306416, an inhibitor of PGF receptor FLT1, to observe the effect of PGF/FLT1 signaling on cell proliferation and migration. We demonstrated that PGF was downregulated in placentas from FGR pregnancies compared with normal controls. The villous expression of PGF was positively correlated with placental and fetal weight. The CpG island inside PGF promoter was hypomethylated without obvious difference in both normal and FGR placentas. However, the higher DNA methylation at another CpG island downstream exon 7 of PGF was demonstrated in FGR placentas. Additionally, we found FLT1 was expressed in trophoblast cells. Inhibition of PGF/FLT1 signaling by a selective inhibitor impaired trophoblast proliferation and migration. In conclusion, our data suggested that the PGF expression was dysregulated, and disrupted PGF/FLT1 signaling in trophoblast might contribute to placenta dysfunction in FGR. Thus, our results support the significant role of PGF in the pathogenesis of FGR.


Introduction

Fetal growth restriction (FGR) or intrauterine growth restriction (IUGR) is characterized as a pathological condition in which a fetus fails to achieve its normal growth potential during the period of intrauterine development (Krishna & Bhalerao 2011, Benton et al. 2016). Currently, FGR remains a non-negligible perinatal problem that would be detrimental to fetuses and neonates. It was reported that FGR was significantly correlated with elevated risk of stillbirth, preterm delivery and perinatal morbidity (Palotto & Kilbride 2006, Miller et al. 2016). Moreover, the incidence of long-term fetal original adult diseases such as cardiovascular disease, neuropsychological dysfunctions and metabolic syndrome (Demicheva & Crispi 2014, Zohdi et al. 2014, Miller et al. 2016) was also greatly increased in FGR newborns.

The pathogenesis of FGR is complicated and still obscure. Numerous studies have suggested that placental and trophoblastic dysfunction was closely associated with the development of FGR (Krishna & Bhalerao 2011, Kovo et al. 2013, Kohli et al. 2017). Some cases of FGR were due to the well-identified etiological factors, such as preeclampsia, intrauterine infection and fetal chromosome abnormality. However, most cases originated from placental insufficiency with unclear insults, which are classified as idiopathic fetal growth restriction (Borg et al. 2015).

Placental growth factor (PGF), a member of the vascular endothelial growth factor (VEGF) family, was initially identified by its important role in angiogenesis of placenta (Carmeliet et al. 2001, Vrachnis et al. 2013).
Further studies uncovered that PGF was involved in a variety of pathological conditions, including diabetic retinopathy, tumorigenesis and atherosclerosis (Carmeliet & Jain 2011, Potente et al. 2011, Dewerchin & Carmeliet 2012). Particularly, the lowered level of PGF in maternal circulation was detected in pregnancies complicated by preeclampsia or FGR (Benton et al. 2012, 2016, Gomez-Roig et al. 2015, Zeisler et al. 2016). Decreased PGF level was recognized as a sign of placental deficiency and associated with adverse perinatal outcomes (Rana et al. 2012, Sibiude et al. 2012). In addition, studies in lung and colon cancer cell lines (Xu & Jain 2007) and endothelial cells (Tudisco et al. 2014) demonstrated that the expression of PGF could be regulated by epigenetic mechanisms.

Besides its role in vasculogenesis and angiogenesis, PGF was also reported to play roles in cancer cell growth and migration. In the present study, we examined the expression of PGF in FGR placenta and explored whether DNA methylation was involved in regulation of PGF in trophoblast. Furthermore, the role of PGF in trophoblast proliferation and migration was also investigated.

### Materials and methods

#### Subjects and clinical samples

Fresh frozen tissue samples (18 from normal pregnancies and 16 from women complicated by FGR) and paraffin embedded blocks (43 from normal pregnancies and 76 from FGR; The summary of clinical characteristics of these subjects was shown in Table 1) of human placentas were retrieved from the archives of Departments of Pathology and Bio-bank of the International Peace Maternity and Child Health Hospital (IPMCH) affiliated to Shanghai Jiao Tong University School of Medicine. Placentas were collected from pregnancies through vaginal delivery or cesarean section with a standard procedure as previously reported (Wu et al. 2016). Idiopathic FGR was defined as fetal growth less than the 10% percentile for gestational age by a combination of prenatal ultrasound assessment (as indicated by abnormal fetal weight estimation, umbilical artery blood flow, amniotic fluid index and ratio of head/abdominal circumference) and postnatal body weight measurement (Borg et al. 2015), with the exclusion of pregnancies with underlying diseases and pregnancies complicated by preeclampsia; gestational diabetes mellitus; intrahepatic cholestasis of pregnancy; hemolysis, elevated liver enzymes and low platelet count (HELLP) syndrome; acute fatty liver of pregnancy; fetal abnormality and intrauterine infection. Simple small for gestational age neonates without prenatal indication were excluded. Normal pregnant women with term delivery and without pregnancy complications were chosen as controls. The ethical approval for this study was issued by IPMCH ethical committee. The corresponding institutional and national guidelines were followed in conducting experiments with human samples. The written informed consents from all participants were obtained before application of placental samples in this study.

### Immunohistochemistry assay

To determine the expression of PGF and TP63α (a marker for proliferative cytotrophoblast) in human placenta tissues, immunohistochemistry (IHC) assay was conducted according to previously described protocol (Wu et al. 2016). Briefly, tissue sections were processed through sequential procedures, including deparaffination, rehydration, antigen retrieval, endogenous peroxidase inactivation and non-specific antigen blocking. Then, the sections were incubated with antibodies against PGF (1:75; #10642-1-AP, Proteintech Group Inc., Rosemont, IL, USA) and TP63α (1:100; #13109, Cell Signaling Technology) at 4°C overnight. Substitution of specific primary antibody with normal rabbit IgG was applied as a negative control. Next, the sections were covered with peroxidase-conjugated polymer goat anti-rabbit IgG antibody from the Dako EnVision Detection System (Dako). Subsequently, immunosignal was visualized by developing 3,3-diaminobenzidine chromogen and counterstaining with Mayer’s hematoxylin. Evaluation of staining signals was performed with the assistance of Leica DM2500 microscope (Leica Microsystems, Wetzlar, Germany) LAS V4.6 software (Leica Microsystems) by two independent pathologists. Discrepancies with some cases were discussed to reach a consensus. Immunostaining intensity was defined by arbitrary scoring: 0, negative; 1+, weak; 2+, moderate and 3+, strong. The area coverage of positive villi was graded as: 0, <1%; 1+, 1–33%; 2+, 33–67%; 3+, >67%. The immunosignal of PGF was calculated by multiplying the two above-mentioned parameters, which results in a composite ‘His-score’ with a minimum value 0 and maximum value 9 (Zhang et al. 2009). For the assessment of TP63α staining, the percentage of positively stained nuclei was counted from at least 1000 trophoblastic nuclei in 5–10 random fields per section as previously reported (Wu et al. 2016).

#### Table 1 Clinical characteristics of the subjects.

<table>
<thead>
<tr>
<th></th>
<th>Normal (n=43)</th>
<th>FGR (n=76)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (years)</td>
<td>30.47 ± 3.210</td>
<td>29.99 ± 3.288</td>
<td>0.444</td>
</tr>
<tr>
<td>BMI of mother</td>
<td>21.37 ± 2.597</td>
<td>21.97 ± 3.360</td>
<td>0.314</td>
</tr>
<tr>
<td>Gravidity</td>
<td>2 (1–4)</td>
<td>1 (1–6)</td>
<td>0.166</td>
</tr>
<tr>
<td>Parity</td>
<td>1 (1–2)</td>
<td>1 (1–2)</td>
<td>0.012</td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>39.00 ± 0.994</td>
<td>36.92 ± 2.490</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>3392.79 ± 342.093</td>
<td>1987.25 ± 437.934</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Placenta weight (g)</td>
<td>554.00 ± 66.083</td>
<td>480.75 ± 78.905</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Placental index</td>
<td>0.16 ± 0.027</td>
<td>0.17 ± 0.041</td>
<td>0.03</td>
</tr>
<tr>
<td>Appgar score at 1 min</td>
<td>10 (7–10)</td>
<td>10 (4–10)</td>
<td>0.021</td>
</tr>
<tr>
<td>Appgar score at 5 min</td>
<td>10 (9–10)</td>
<td>10 (6–10)</td>
<td>0.146</td>
</tr>
</tbody>
</table>

Values are given as median (range), mean ± s.d., or N (percent). BMI, body mass index; placental index, placenta weight/birth weight.

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ELISA assay

Fresh frozen placental tissues were disrupted in RIPA lysis buffer by homogenization. The supernatant was subjected to ELISA for detecting PGF level using human PGF QuantiKine ELISA Kit from R&D Systems according to manufacturer’s instruction. PGF content in placenta was calculated by normalizing to total protein concentration.

DNA methylation analysis by bisulfite sequencing

Genomic DNA from placental tissues was isolated using a TIANamp Genomic DNA Kit (Tiangen Biotech, Beijing, China). For the methylation analysis, 500 ng of genomic DNA was subjected to sodium bisulfite treatment and purification by using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA, USA) according to the manufacturer’s protocol. Amplification of target region 1 at PGF promoter (CpG Island 1, Fig. 2) using KAPA2G Robust HotStart ReadyMix (Kapa Biosystems, Woburn, MA, USA) was applied with a nested PCR method as described previously (Tudisco et al. 2014). The primers for amplification of CpG island 1 were used as previously reported (Tudisco et al. 2014). Target region 2 at PGF downstream region (CpG Island 2, Fig. 2) was amplified using a conventional 3-step PCR procedure (95°C for 30 s, 56°C for 30 s and 72°C for 25 s – 35 cycles). For PCR amplification of CpG island 2, primers for BSP were designed using MethPrimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) and listed as follows: forward 5′-GTTGATTTAGTGGGTGTGTTTTT-3′ and reverse 5′-ATCAACCATTTCACCTTCTTAAAC-3′. PCR products were purified and cloned into the pGM-T vector (Tiangen Biotech, Beijing, China). The sequence of 10 clones for each placental sample was determined by DNA sequencing.

Cell culture and transfection

Human choriocarcinoma cell lines JAR and JEG3 were from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). JAR and JEG3 cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin/streptomycin in a Thermo Scientific incubator (37°C, 5% CO2). BeWo cells obtained from Chinese Academy of Medical Sciences & Peking Union Medical College (Beijing, China) were maintained in 1:1 mixture of DMEM and Ham’s F-12 medium (DMEM/F12; Invitrogen) supplemented with 15% FBS and 1% penicillin/streptomycin. The immortalized trophoblast cell line (HTR8/SVneo), a kind gift from Dr Charles H Graham (Queen’s University at Kingston), was maintained in RPMI-1640 supplemented with 10% FBS.

Scramble siRNA or siRNA duplex targeting human PGF was purchased from GenePharma (Shanghai, China). PGF siRNA sequences were as follows: siPGF-1 (sense) 5′-CCAUGCAGCUCUCCAAAGAUdTdT-3′, (antisense) 5′-AUCUUUAAGGAGCUGCAUGGdTdT-3′; siPGF-2 (sense) 5′-GACGUUUCUCUCAGCACGUUdTdT-3′, (antisense) 5′-AACGUGCUGAGAGACGUCdTdT-3′. Transient transfection with siRNA oligonucleotides (50 nM) was performed using HiPerFect Transfection Reagent (Qiagen) according to the manufacturer’s instruction. Forty-eight hours after transfection, cells were harvested by trypsin digestion and subjected to quantitative PCR analysis of PGF expression and cell proliferation assays.

RNA isolation, reverse transcription and real-time quantitative PCR assay

Placental tissues were dissected into small pieces and homogenized in Trizol reagent (Invitrogen). Isolation of total RNA from tissues and cells was conducted using standard methods. The RNA concentration and purity were analyzed using a spectrophotometer. The cDNA library was synthesized using a reverse transcription kit (TaKaRa, Dalian, China) according to the manufacturer’s instructions. The gene-specific primers used for RT-qPCR analysis are listed in Table 2.

Table 2 Primers for RT-qPCR assay.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Alias</th>
<th>Accession number</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGF</td>
<td>PGF</td>
<td>NM_002632.5</td>
<td>F: TGTCACCATGCAGCTCTCTAAA, R: TGGCGATCTGCTGGTCTCTG</td>
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<tr>
<td>RNA18S5</td>
<td>18S rRNA</td>
<td>NR_146119.1</td>
<td>F: CAGGCCACCCAGGATTAGCAGCA, R: TATGACCAGCGCCGGTCTG</td>
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<tr>
<td>FLT1</td>
<td>VEGFR1</td>
<td>NM_002019.4</td>
<td>F: GAAACCCGATACTCCGGACAGT, R: CCCTGTTGTGCTTTTGGG</td>
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<tr>
<td>VEGF</td>
<td>VEGF</td>
<td>NM_001025366.2</td>
<td>F: CTGTCTATGCTCTGCGGCCGG, R: AGACGAGTCTCTGTGTTTGC</td>
</tr>
<tr>
<td>KDR</td>
<td>VEGFR2</td>
<td>NM_002253.2</td>
<td>F: GTGATGCGAGACAACGTAGAG, R: CATGTTGGTGATCCTAAGAAAGCA</td>
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<tr>
<td>CCNA2</td>
<td>Cyclin A2</td>
<td>NM_001237.4</td>
<td>F: CACGGGTGGCTCACTAACAGAGA, R: CATCTGCTGCCTGCTGAGTATGTC</td>
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<tr>
<td>CCNB1</td>
<td>Cyclin B1</td>
<td>NM_031966.3</td>
<td>F: TTTGCGACATGTTGCAAAAATGTC, R: ATAGGGTCAGCGCGAAAGTTTT</td>
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<tr>
<td>CCND1</td>
<td>Cyclin D1</td>
<td>NM_053056.2</td>
<td>F: AACTTGGCTGCTGACGACGCAG, R: CATCCGAAGACAGCAAGACTCTC</td>
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<td>CCNE1</td>
<td>Cyclin E1</td>
<td>NM_001238.3</td>
<td>F: GTCTCAGCGCAGCAGCTC, R: ACTCCGCAAGACAGCAAGCAGCTC</td>
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<tr>
<td>CDKN2A</td>
<td>p16INK4A</td>
<td>NM_000077.4</td>
<td>F: ACTTCAGGGGTGCCACATTC, R: CGACCCTGTCCCTCAATCC</td>
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<tr>
<td>CDPN1A</td>
<td>p21WAF1/CDKI</td>
<td>NM_001291549.1</td>
<td>F: FGGATGCCGTCAGCAAG, R: GCTCAGCGGCGCGAGCTG</td>
</tr>
</tbody>
</table>

www.reproduction-online.org
protocol as previously described (Wu et al. 2016). And then, cDNA was reverse-transcribed (RT) from purified RNA using 5X RT MasterMix from ABM Inc. (Richmond, BC, Canada). To determine gene expression profiles, real-time quantitative PCR assay (qPCR) was performed using RT² SYBR Green qPCR Mastermixes (Qiagen). PCR was monitored in a StepOnePlus Real-Time PCR System (Applied Biosystems) with an initial denaturation step (95°C for 2 min) and 40 cycles of two-step PCR (95°C for 15 s, 60°C for 30 s). Sequences of primer sets for qPCR are shown in Table 2. Relative expression level of target gene with normalization to an endogenous reference (18S rRNA) was calculated by the 2−ΔΔCt method.

Cell proliferation assays

Evaluation of cellular proliferative activity was carried out using the Cell Counting Kit-8 (Dojindo, Kamimashiki-gun, Kumamoto, Japan) as previously described (Wu et al. 2014). In brief, cells were dispersed by trypsin digestion and seeded into a 96-well plate (5000 cells per well). Cells were cultured at 37°C under 5% CO2, until further analysis. At an interval of 12 h or 24 h, viable cells were quantified by incubation with the WST-8 reagent for 1 h and subsequently measuring the optical density (OD) at 450 nm in a SpectraMax M3 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

Transwell cell migration assay

Trophoblast cell motility was examined using 24-well transwell chambers with 8 μm pore size membrane (Corning) as previously described (Wu et al. 2014). Briefly, JAR and JEG-3 cells (0.5–1.5×105 cells/well) resuspension in serum-free medium were seeded into transwell chamber, the outer face of which was immersed in DMEM containing 10% FBS. After 18–24 h incubation at 37°C, residual cells in the inner face of transwell chamber were removed by gentle scrape. And cells adherent on the outer surface were fixed in 4% paraformaldehyde and further stained with 0.1% crystal violet. Image was taken under the Leica DM2500 microscope (Leica Microsystems). For quantification, cell nuclei were labeled with fluorescent dye Hoechst 33342 and the number of migrated cell from six random fields/samples was counted under a Leica fluorescence microscope (Leica Microsystems).

Statistical analysis

Statistical analysis of experimental and clinical data was applied on the GraphPad Prism Version 5.0 (GraphPad Software) and Statistical Package for Social Science 16.0 (SPSS). Numeric data were analyzed by two-tailed Student’s t test, two-way ANOVA and Mann-Whitney test. Data were expressed as mean ± S.E.M. from independent groups. Chi-square test was applied for categorical values. Correlation analysis of parameters was conducted using Pearson correlation or Spearman’s correlation test. The P value less than 0.05 was defined as statistical significance.

Results

PGF was downregulated in idiopathic FGR placenta

Firstly, we analyzed the expression of PGF in human placentas from normal and FGR pregnancies by RT-qPCR assay and ELISA. It was demonstrated that both mRNA and protein levels of PGF in placental tissues were significantly lower in FGR group compared with normal pregnancies (Fig. 1A and B; both P < 0.05). Then, through IHC assay, we observed that PGF was primarily localized to syncytiotrophoblast layer and intravillous mesenchyma of placental villi (Fig. 1C). In addition, summarized His-score of PGF by IHC assay demonstrated that His-score in FGR placentas was much lower than that in placentas from normal pregnancies (Fig. 1D; P < 0.001). We further analyzed the correlation among His-score, fetal weight and placental weight by Pearson’s Correlation and Spearman’s Correlation tests. As shown in Fig. 1E, F, G and Table 1, fetal weight and placental weight were both decreased in FGR placentas (both P < 0.001). And these two parameters were positively correlated (P < 0.001) as expected. Moreover, significant correlation was found between His-score and fetal weight or placental weight with correlation coefficient of 0.584 or 0.563 respectively (Table 3; both P < 0.001). Taken together, these findings indicated that the protein and mRNA expression of PGF is decreased in placentas of FGR pregnancies.

Promoter methylation did not contribute to PGF downregulation in FGR placentas

To investigate the potential mechanism for reduced PGF expression in FGR placentas, the methylation profile of CpG islands within human PGF gene was examined in placental tissues. A CpG island in promoter region overlapping the exon 1 on PGF gene (Fig. 2A; CpG Island 1, −114/+390) was previously reported (Tudisco et al. 2014). DNA methylation status was analyzed by bisulfite sequencing. Ten clones for each sample were analyzed, and the universal hypomethylation at a total of 60 CpG sites was present in both groups. There is no obvious difference in FGR placentas compared with the normal control (Fig. 2B and C; P = 0.198). On the other hand, application of a DNA methyltransferase inhibitor (5-Aza-2'-deoxycytidine; 5-AZA) for 48 h increased PGF mRNA expression to 2.16- and 1.92- folds in two trophoblastic cell lines JAR and BeWo respectively (Fig. 2D and E). Further bioinformatic analysis of PGF gene suggested that another possible CpG island (CpG Island 2, +13,935/+14,154) was harbored downstream exon 7 of PGF gene (Fig. 2A). We found that CpG sites at this region were moderately methylated in all samples and total methylation level was slightly higher in FGR placentas compared with placentas from normal.
PGF was downregulated in FGR placenta

The endogenous expressions of PGF and its receptor FTL1 in trophoblast cell lines were examined first. We found that PGF and FTL1 were abundantly expressed in JAR, JEG3 and BeWo cells (Fig. 3A and B). However, the HTR8/SVneo cell, which is an immortalized extravillous trophoblast cell line, expressed extremely low levels of PGF and FTL1. Although VEGFA was slightly expressed, KDR, the major receptor for VEGF, was non-detectable in these cell lines (Fig. 3C and D). To further study the role of PGF in trophoblasts, JEG3 cells were transfected with two siRNA duplexes against PGF or scramble siRNA (Scr). As shown, the level of PGF mRNA was markedly knocked down by PGF siRNA transfection (Fig. 4A). The growth rates of PGF-knockdown cells (siPGF-1 and siPGF-2) were slightly lower than the Scr control (Fig. 4B). Moreover, administration of a selective FTL1 inhibitor ZM-306416 moderately inhibited cell proliferation (Fig. 4C; P < 0.001). The expressions of cell cycle regulators were further analyzed by RT-qPCR assay. We observed that genes involved in cell cycle progression (CCNA2, CCNB1, CCND1 and CCNE1) were significantly downregulated, while cell cycle inhibitors (p16 and p21) were not obviously changed in ZM-306416-treated cells compared with DMSO-treated control cells (Fig. 4D). Interestingly, the expression of PGF was also evidently decreased upon ZM-306416 treatment, suggesting a possible positive feedback regulation of PGF expression in PGF/FTL1 signaling pathway. Additionally, immunostaining with TP63α, a well-featured marker for cytotrophoblasts (Ramalho et al. 2006, Zhang et al. 2009, Li et al. 2014) in placenta, demonstrated that the number of cytotrophoblasts was evidently decreased in placentas from FGR pregnancies compared with those from normal pregnancies (Fig. 3E and F). The lower proportion of TP63α-positive nuclei indicated a possible defect in cytotrophoblast (as trophoblastic stem cell) proliferation in FGR placentas. Taken together, these data indicated that PGF/FTL1 signaling might participate in regulation of trophoblast cells proliferation.

Table 3  Correlation tests among PGF His-score, placenta weight and birth weight.

<table>
<thead>
<tr>
<th>Correlation test</th>
<th>Parameters</th>
<th>Correlation coefficient</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pearson’s correlation</td>
<td>Placenta weight</td>
<td>0.873</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Birth weight</td>
<td>0.873</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Spearman’s correlation</td>
<td>Placenta weight</td>
<td>0.584</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>PGF His-score</td>
<td>0.566</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Birth weight</td>
<td>0.563</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Inhibition of PGF signaling affected trophoblast growth

The endogenous expressions of PGF and its receptor FTL1 in trophoblast cell lines were examined first. We found that PGF and FTL1 were abundantly expressed in JAR, JEG3 and BeWo cells (Fig. 3A and B). However, the HTR8/SVneo cell, which is an immortalized extravillous trophoblast cell line, expressed extremely low levels of PGF and FTL1. Although VEGFA was slightly expressed, KDR, the major receptor for VEGF, was non-detectable in these cell lines (Fig. 3C and D). To further study the role of PGF in trophoblasts, JEG3 cells were transfected with two siRNA duplexes against PGF or scramble siRNA (Scr). As shown, the level of PGF mRNA was markedly knocked down by PGF siRNA transfection (Fig. 4A). The growth rates of PGF-knockdown cells (siPGF-1 and siPGF-2) were slightly lower than the Scr control (Fig. 4B). Moreover, administration of a selective FTL1 inhibitor ZM-306416 moderately inhibited cell proliferation (Fig. 4C; P < 0.001). The expressions of cell cycle regulators were further analyzed by RT-qPCR assay. We observed that genes involved in cell cycle progression (CCNA2, CCNB1, CCND1 and CCNE1) were significantly downregulated, while cell cycle inhibitors (p16 and p21) were not obviously changed in ZM-306416-treated cells compared with DMSO-treated control cells (Fig. 4D). Interestingly, the expression of PGF was also evidently decreased upon ZM-306416 treatment, suggesting a possible positive feedback regulation of PGF expression in PGF/FTL1 signaling pathway. Additionally, immunostaining with TP63α, a well-featured marker for cytotrophoblasts (Ramalho et al. 2006, Zhang et al. 2009, Li et al. 2014) in placenta, demonstrated that the number of cytotrophoblasts was evidently decreased in placentas from FGR pregnancies compared with those from normal pregnancies (Fig. 3E and F). The lower proportion of TP63α-positive nuclei indicated a possible defect in cytotrophoblast (as trophoblastic stem cell) proliferation in FGR placentas. Taken together, these data indicated that PGF/FTL1 signaling might participate in regulation of trophoblast cells proliferation.
Figure 2 The effect of DNA methylation of PGF gene on the expression of PGF was analyzed in normal controls and FGR pregnancies. 
(A) Schematic diagram of the CpG island 1 (−114/+390 refer to transcriptional start site (indicated by the arrow)) located in the proximal PGF promoter region and another potential island (CpG island 2, +13,935/+14,154) downstream human PGF gene (CpG islands were shown as filled rectangle). (B and C) Methylation profile of genomic DNA at CpG island 1 in PGF promoter after bisulfite treatment was analyzed. (B) Ten clones for each of normal and FGR placentas were analyzed. (Empty circles: unmethylated; filled circles: methylated). (C) The percentage of methylated DNA at PGF promoter as analyzed by bisulfite sequencing was summarized in both groups (n=12 for each group; P=0.198).

(D and E) JAR (D) and BeWo (E) cells treated with a DNA methyltransferase inhibitor (5-Aza-2′-deoxycytidine; 5-AZA, 5 μM) for 48 h were subjected to RT-qPCR assay for detecting PGF mRNA level. (F and G) Methylation status of genomic DNA at CpG island 2 downstream PGF gene was analyzed by bisulfite sequencing. (F) Methylation statuses of CpG sites at island 2 in representative samples of normal and FGR placentas were shown. (G) DNA methylation level at CpG island 2 was analyzed (n=12 for each group; *P<0.05).
Inhibition of PGF/FLT1 signaling blunted motility of trophoblasts

We further explored whether PGF signaling modulated the migration of trophoblastic cells. DMSO or 10μM ZM-306416-treated JAR and JEG-3 cells were subjected to transwell cell migration assay. We found that the motility of JAR and JEG-3 cells were substantially inhibited in response to ZM-306416 treatment (Fig. 5A). As shown, inhibition of PGF/FLT1 signaling by ZM-306416 markedly attenuated the migration of trophoblast cells.

Figure 3 PGF and FLT1 were expressed in trophoblast cell lines. The mRNA expressions of PGF (A) and its receptor FLT1 (B) as well as the mRNA expressions of VEGFA (C) and its major receptor KDR (VEGFR2) (D) were determined by RT-qPCR assay in trophoblast cell lines (JAR, JEG3, BeWo and HTR8/SVneo) and placental tissues (1st trimester and term). ND, not detected.

Figure 4 PGF/FLT1 signaling was involved in trophoblast growth regulation. (A and B) JEG3 cells were transiently transfected with PGF siRNA (siPGF-1 and siPGF-2) or scramble siRNA duplex (Scr). The mRNA expressions of PGF were detected by RT-qPCR (A: ***P<0.001, Student’s t test) and the proliferation rate was determined by CCK-8 assay (B: **P<0.01, ***P<0.001, two-way ANOVA). (C) CCK-8 cell proliferation assays were applied to examine JAR cell growth rate after DMSO or ZM-306416 (10μM) treatment at the indicated time points (**P<0.01, ***P<0.001, two-way ANOVA). (D) JAR cells treated with DMSO or ZM-306416 for 24h were subjected to determination of CCNA2, CCNB1, CCND1 and CCNE1, and p16 and p21 mRNA levels by RT-qPCR assay. (**P<0.05, ***P<0.001; ns, not significant). (E) Placentas from normal term and FGR pregnancies were immunostained with cytotrophoblast cell marker TP63α (original magnification 400×, bar: 50 μm). (F) Statistical analysis of TP63α immunoreactivity in placentas was applied by determining the proportion of positive nuclei from total trophoblast nuclei (n = 43 for normal, n = 76 for FGR, ***P<0.001, Mann–Whitney test).
of villous PGF produced by trophoblasts was also decreased in placentas from pregnancies complicated by idiopathic fetal growth restriction, which may associate with placental dysfunction.

Moreover, significant correlation was found between the level of villous PGF and fetal weight as well as placental weight. PGF was identified as a major angiogenic factor in placenta, which plays a key role in placenta and fetal development (Vrachnis et al. 2016). PGF secreted by villous trophoblasts was deemed as the predominant source of PGF in placenta (Taylor et al. 1997). Most importantly, dysregulated PGF expression in placenta may compromise the process of angiogenesis and vascular remodeling under pathophysiological condition (Potente et al. 2011, De Falco 2012). Indeed, placental vascular lesion was commonly observed in FGR placentas (Kovo et al. 2010). The pathological changes of villous vasculature were associated with hypoxic phenotype in FGR placentas (Krishna & Bhalerao 2011, Kovo et al. 2013). Decline in oxygen supplementation could lead to impaired placental growth and retarded fetal development. Coincidentally, we observed that there were 42.1% FGR pregnancies with fetal distress (linked to intrauterine hypoxia) in our study cohort.

The expression of PGF in placenta was regulated by many factors, such as cAMP/PKA, hypoxia-inducible factor-1α (HIF-1α) (Depoix et al. 2011, Tudisco et al. 2014). It was well accepted that the level of PGF together with VEGF in placenta was primarily modulated by oxygen concentration (Ahmed et al. 2000). Generally, high oxygen tension increased PGF expression, while low oxygen concentration reduced PGF and increased VEGF (Khaliq et al. 1999, Ahmed et al. 2000). However, it was also reported by an in vitro study that expression of PGF was strongly induced by HIF-1α under hypoxia in endothelial cells (Tudisco et al. 2014). The role of hypoxia and HIF-1α in PGF regulation in trophoblast still requires comprehensive investigation. By IHC assay, we found that the expression of PGF protein was substantially decreased in placentas’ villi from FGR pregnancies compared with the normal controls. The major reason for the reduction of PGF might be that the production of PGF by dysfunctional syncytiotrophoblasts was lessened. However the raised level of soluble fms-like tyrosine kinase-1 (sFlt1), a soluble receptor for PGF, was associated with lowered circulating level of free PGF in pregnancies with preeclampsia and FGR (Holme et al. 2016, Zeisler et al. 2016). Nevertheless, whether the increased sFlt1 may compromise the PGF expression in placental villous parenchyma still needs to be elucidated.

Both normal and FGR placentas displayed hypomethylation in PGF promoter with no obvious difference of methylation level in our study. A previous study also demonstrated that hypomethylation at PGF promoter was present in cultured endothelial cells, which
was not affected by hypoxic conditions (Tudisco et al. 2014). In contrast, another study showed that PGF promoter was hypermethylated, which contributed to PGF downregulation in lung and colon cancer tissues and cell lines (Xu & Jain 2007). These studies indicated that the methylation profile of the PGF promoter may depend on distinct cellular contexts. Interestingly, we discovered another potential CpG island downstream exon 7 of PGF gene locus, adjacent to 3'-UTR regions. CpG sites at this region (CpG island 2) were hypermethylated. And the differential methylation level at this region might contribute to PGF downregulation in FGR placentas compared with normal placentas. The CpG island 2 might be located inside a distal enhancer region of PGF gene, and the methylation at distal CpG islands may have an influence on chromatin structure and modulate PGF transcription. Moreover, treating with a DNA methyltransferase inhibitor, 5-Aza-2'-deoxycytidine, significantly enhanced PGF expression in trophoblastic cell lines JAR and BeWo. Our data suggested that distal DNA methylation might regulate PGF expression in trophoblasts through a direct or indirect epigenetic mechanism, which requires further studies.

PGF was primarily recognized because of its ability to induce proliferation and migration of endothelial cells in vascular formation (Maglione et al. 1991, De Falco 2012, Dewerchin & Carmeliet 2014). Besides its role in angiogenesis, PGF could also regulate cell growth and migration in various cell types. Recent studies have revealed that the expression level of PGF was correlated with cancer cell growth and tumor progression in a variety of cancers, including endometrial carcinoma (Coenegrachts et al. 2013), melanoma (Pagani et al. 2016), ovarian cancer (Song et al. 2016), non-small cell lung cancer (Zhang et al. 2015) and renal cell carcinoma (Bessho et al. 2013). In addition, overexpression of PGF promoted cancer cell migration, and cancer cell invasion as well as metastasis in colorectal cancer and pancreatic carcinoma (Fischer et al. 2007, Coenegrachts et al. 2010). Therefore, we determined the role of PGF and its receptor FLT1 (Yao et al. 2011, Wei et al. 2013) in trophoblast proliferation and migration. We demonstrated here that FLT1 and PGF were abundantly expressed in cytotrophoblast-like choriocarcinoma cells, JAR, JEG and BeWo. These cells could differentiate to multinucleated syncytiotrophoblast-like cells with synthesis and secretion function in vitro (Kohli et al. 2017). As expected, attenuation of PGF/FLT1 signaling by a selective inhibitor ZM-306416 led to trophoblast dysfunction, as evidenced by impaired proliferation and migration. Moreover, we found the number of TP63α-positive cytotrophoblasts in FGR placenta was decreased, which may result from defective proliferation in cytotrophoblasts. However, PGF and FLT1 were expressed at extremely low level in an immortalized extravillous trophoblastic cell line, HTR8/SVneo. A previous study also reported that PGF could not regulate the motility of extravillous trophoblast (Lash et al. 1999). Thus, the current research could not support the role of PGF/FLT1 signaling in the modulation of extravillous trophoblast activity, such as spiral arterial remolding in placenta. Based on the above-mentioned findings, we supposed that PGF produced by syncytiotrophoblasts might involve in syncytiotrophoblast–cytotrophoblasts crosstalk and contribute to the proliferation and differentiation of cytotrophoblasts in placenta. Although we took advantage of choriocarcinoma cells in functional assays, we had to admit that there were limitations associated with using these cells in studying the role of PGF in FGR. These cells are more active in proliferation and migration than non-neoplastic trophoblastic cells. Further studies with animal model and FGR term placenta explant culture would be beneficial for better understanding the effect of compromised PGF/FLT1 signaling on the development of FGR.

In summary, this study demonstrated that the expression of PGF was downregulated in FGR placentas. The expression of PGF in placenta was correlated with placental and fetal growth. Although the expression of PGF was not associated with promoter hypermethylation, distal DNA methylation might regulate PGF expression through a direct or indirect manner. PGF/FLT1 signal modulated cell proliferation and motility in trophoblasts, which are crucially important for the development and function of placenta. Our data suggested that dysregulated PGF expression and disrupted PGF/FLT1 signaling in trophoblast might be involved in malfunction of FGR placenta. PGF is thus supposed to be a potential clinical indicator for monitoring FGR.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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