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Phosphoglucomutase 5 gene transcripts are expressed by the human placenta and differentially regulated in placental dysfunction

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The placenta plays an essential role facilitating nutrient, gas and waste exchange between the maternal and fetal systems for optimal fetal growth. When placental development is impaired and the placenta dysfunctional, serious pregnancy complications such as fetal growth restriction and preeclampsia may arise. Previously, phosphoglucomutase-5 (PGM5) transcripts were found to be highly elevated in the blood of patients whose pregnancies were complicated by fetal growth restriction and preeclampsia. As both conditions feature placental insufficiency, here we aimed to characterise PGM5 levels in the healthy and dysfunctional placenta. *PGM5* expression was detectable in all placental samples across gestation, in cases of preterm preeclampsia, fetal growth restriction and controls. *PGM5* mRNA expression was significantly downregulated in the pathological placentas compared to controls, but PGM5 protein production was not dysregulated. Isolated cytotrophoblast and placental explant tissue exposed to hypoxia (modelling placental dysfunction) demonstrated significantly increased PGM5 expression, but again did not change protein levels. Silencing *PGM5* expression under hypoxic conditions in primary cytotrophoblast did not alter anti-angiogenic sFLT-1 secretion but increased expression of multiple genes associated with cell growth, apoptosis and oxidative stress, whilst also increasing cell viability. Expression of PGM5 in all placental samples assessed suggests that PGM5 has functions in the placenta. However, further investigation could be performed to explore the discrepancies in protein and mRNA expression, as well as the precise function of PGM5 in the placenta, and whether altered PGM5 levels may be important for placental development.

The placenta is a highly specialised organ essential to human pregnancy. It acts as the interface between the maternal and fetal systems, facilitating the supply of nutrients and oxygen to the fetus whilst removing waste products, for optimal fetal growth. As such, placental development is a key determinant of a successful pregnancy. When placental development and function is impaired, pregnancy complications such as fetal growth restriction (FGR) and preeclampsia may arise[1,2]. These complications can result in deficient fetal growth and development, or in severe cases lead to fetal demise.

FGR refers to when a fetus does not grow to its expected potential for its gestational age, and can occur when the fetus does not receive sufficient nutrients to support their growth due to pathology (often termed small for gestational age, < 10th centile). Preeclampsia is relatively a more complex condition affecting up to 7% of all pregnancies worldwide[3], characterised by both a maternal and uteroplacental phenotype. It is clinically defined as new onset of hypertension in the second half of pregnancy, accompanied by one or more of proteinuria, uteroplacental insufficiency and/or major organ dysfunction (neurological, hepatic or haematological)[4]. In preeclampsia, the dysfunctional placenta releases excess levels of anti-angiogenic and pro-inflammatory factors causing widespread maternal vascular dysfunction, hypertension, and major organ injury, the clinical symptoms of disease. While the implications of FGR are felt mostly by the developing fetus, the combination of maternal and fetal effects in preeclampsia can impact the wellbeing of both the birthing individual and child. Despite affecting so many, there are few treatment options currently available to treat FGR and preeclampsia. As such, there is a vital need for the development of strategies to prevent and treat these serious conditions of pregnancy.

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The placenta, being a central player in disease pathology is an obvious target for treatments – as improving placental function could improve fetal growth. Unfortunately, there is still much we do not understand about this organ, though its role is essential for human life. There is thus a need to improve our understanding of the mechanisms driving placental development over gestation, and the mechanisms that drive placental dysfunction.

Previously, our team discovered that phosphoglucomutase-5 (PGM5) transcripts were highly elevated in the circulation of individuals whose pregnancies were complicated by early onset fetal growth restriction, with and without preeclampsia[5]. It was postulated that the differential changes observed in PGM5 transcripts in the circulation could help detect babies in distress with fetal hypoxia[6]. However, in this original study we did not investigate the origin of the *PGM5* transcripts. Here, in the present study, we set out to determine whether the placenta was the likely tissue source. We specifically aimed to measure PGM5 protein and mRNA levels in healthy control human placenta and placenta from pregnancies complicated by preeclampsia and fetal growth restriction (pathological). Furthermore, we aimed to determine whether silencing *PGM5* transcripts in the placenta in normal oxygen and hypoxic conditions had downstream effects.

PGM5 is one of five proteins making up the phosphoglucomutase family, most closely related to PGM1, and is found only in vertebrates[7,8]. Studies suggest that unlike its family members, PGM5 has no phosphoglucomutase activity – instead its coded protein, aciculin acts as a cytoskeleton protein, co-localised with dystrophin or utrophin[9,10]. However, its role in placental development has not been previously investigated. Here we use primary human placenta to characterise PGM5 expression over gestation and investigate its function in the human placenta.

Methods

Placental tissue collection

Ethical approval was obtained for this study from the Mercy Health Human Research Ethics Committee (R11/34). Women presenting to the Mercy Hospital for Women (Heidelberg, Victoria) gave informed, written consent for the collection of their placenta and umbilical cord. Women presenting to the Broadmeadows Health Service (Broadmeadows, Victoria; HREC/18/Austin/44) gave informed, written consent for the collection of conceptus samples at surgical termination of pregnancy. All experiments were performed in accordance with institutional guidelines and regulations.

Pathological placental tissue was collected from pregnancies complicated by early onset preeclampsia (delivery ≤ 34 weeks' gestation) and/or preterm fetal growth restriction (delivery ≤ 34 weeks' gestation). Preeclampsia was diagnosed by an obstetrician in accordance with American College of Obstetricians and Gynaecologists (ACOG) guidelines[11]. Fetal growth restriction was defined as < 10th centile according to Australian population charts[12]. Cases associated with congenital infection, chromosomal or congenital abnormalities or multiple pregnancies were excluded.

Placenta tissue for control purposes was collected from pregnancies at term (delivered via caesarean section between 37 and 40 weeks' gestation) and early preterm (delivered ≤ 34 weeks' gestation; due to non-confounding complications defined as clinical conditions that serve as appropriate controls for placenta, such as placenta previa, premature rupture of membranes, maternal illness[13]). Placenta was collected from normotensive pregnancies where a fetus of normal birth weight centile (> 10th centile relative to gestation) was delivered. Cases associated with placental infection/chorioamnionitis, chromosomal or congenital abnormalities, multiple pregnancies or any other placental pathology were excluded.

First trimester placental tissue was obtained from conceptus material collected at surgical terminations of singleton pregnancies (7–11 weeks gestation) under general anaesthesia via curettage and/or aspiration (according to the surgeon's preference). Placental tissue was identified and dissected from conceptus material, then washed in phosphate buffered saline (PBS). Placental tissue was transferred to RNAlater for 48 h, then snap frozen and stored at − 80 °C for subsequent analysis.

Term and preterm placental tissue were collected within 30 min of delivery. Tissue was randomly sampled from four separate sites of the placenta, washed in ice-cold PBS and preserved with RNAlater Stabilization Solution (Invitrogen™, Waltham, USA) for 48 h. Samples were stored at − 80 °C for RNA and protein extraction. Patient characteristics are presented in Supplementary Tables 1–3. Sample cohorts utilised for in vitro experiments were balanced for fetal sex.

Collection and culture of placental explants

Explant dissection was performed on placentas obtained from normal term pregnancies (> 37 weeks gestation) at elective caesarean section. Maternal and fetal surfaces were dissected and removed. Three pieces of placenta totalling 15 mg were placed in each well of a 24-well plate. Wells were cultured with Gibco™ Dulbecco's Modified Eagle Medium (DMEM; ThermoFisher Scientific, Scoresby, VIC, Australia) supplemented with 10% fetal calf serum (FCS; Sigma-Aldrich, St Louis, MO, USA) and 1% Antibiotic Antimycotic (AA; Life Technologies, Carlsbad, CA, USA). Explants were cultured under 8% O_2, 5% CO_2 at 37 °C overnight (16–18 h). After replacement with fresh media (DMEM, 10% FCS, 1% AA), explant tissue was cultured at 37 °C for 48 h under 8% O_2 (normoxic conditions) or 1% O_2 (hypoxia). Following this, explant tissue was weighed, snap frozen and stored at − 80 °C for subsequent analysis.

Primary cytotrophoblast isolation and hypoxia treatment

Human primary cytotrophoblasts were isolated from normal term placentas from elective caesarean section as previously described[14]. The cells were plated in media (DMEM, 10% FCS, 1% AA) on fibronectin (10 µg/mL; BD Bioscience, USA) coated culture plates. Viable cells were incubated under 8% O_2, 5% CO_2 at 37 °C overnight to equilibrate. After replacement with fresh media, cytotrophoblasts were cultured at 37 °C for 24 h under 8% or 1% O_2. Following this, cells were collected for RNA extraction.

Silencing PGM5 in primary cytotrophoblast cells

Short interfering (si)RNA against *PGM5* (M-008537-01-0005; Dharmacon, Lafayette, California, USA) or negative control (Qiagen, Hilden, Germany) were complexed at 10nM with lipofectamine (RNAiMax; Invitrogen, Waltham, WA, USA) diluted 1:100 in Opti-MEM media (ThermoFisher Scientific) for 20 min at room temperature. The siRNA complex was added to pre-equilibrated cytotrophoblasts in media (DMEM/10% FCS) in a dropwise manner. Cells were incubated for 48 h at either 8% or 1% oxygen tension prior to collection of media and cell lysates for subsequent analysis.

MTS cell viability assay

Cell viability was assessed following siRNA treatment using the MTS assay, CellTiter 96-AQueous One Solution (Promega, Madison WI) according to manufacturer instructions.

Real time polymerase chain reaction (RT-PCR)

RNA was extracted from tissue, explants and primary trophoblasts using the Qiagen RNeasy Mini Kit following the manufacturer's instructions. RNA was quantified using a Nanodrop 2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). Extracted RNA was converted to cDNA using the Applied Biosystems High-Capacity cDNA Reverse Transcription Kit, as per manufacturer guidelines on the iCycler iQ5 (Biorad). Taqman gene expression assays (Life Technologies, Carlsbad, USA) were used to quantify mRNA expression of *PGM5 (*Hs00222671_m1), *BAX* (Hs00180269_m1), *BCL2* (Hs00608023_m1), *EGFR* (Hs01076078_m1), *IGF2* (Hs04188276_m1), *NOX4* (Hs00418356_m1). The stability of reference genes was confirmed for data analysis dependent on sample type: *YWHAZ* (Hs01122454_m1) for trophoblast cells, or *TOP1* (Hs00243257_m1) and *CYC1* (Hs00357717_m1) for explants and placental tissue. Taqman RT-PCR was performed on the CFX384 (Biorad) with the following run conditions: 50 °C for 2 min; 95 °C for 10 min, 95 °C for 15 s, 60 °C for 1 min (40 cycles).

The sFLT-1 splice variants *i13* and *e15a* were measured in a SYBR PCR with SYBR Green Master mix (Applied Biosystems) using primers specific for each variant. The primers for *i13* were 5'-ACAATCAGAGGTGAGCAC TGCAA-3' (forward) 5'-TCCGAGCCTGAAAGTTAGCAA-3' (reverse), for *e15a* 5-CTCCTGCGAAACCTCA GTG-3' (forward) 5'-GACGATGGTGACGTTGATGT-3' (reverse) and for *YWHAZ* 5'-GAGTCATACAAAGA CAGCACGCTA-3' (forward) 5'-TTCGTCTCCTTGGGTATCCGATGT-3' (reverse). The SYBR PCR was run on the CFX384 (Biorad), with 40 cycles of 95 °C for 21 s, then 60 °C for 20 min. All data were normalized to an appropriate reference gene as an internal control and calibrated against the average Ct of the control samples. All cDNA samples were run in duplicate.

Western blot

Protein lysates were extracted from placental tissue using RIPA lysis buffer containing proteinase and phosphatase inhibitors (Sigma Aldrich). Protein concentrations were determined using Pierce BCA Protein Assay Kit (ThermoFisher Scientific). Protein lysates (20 µg) were separated on 12% gels before transfer to PVDF membranes (Millipore; Billerica, MA, USA). Membranes were blocked with 2% bovine serum albumin (BSA; Sigma-Aldrich) prior to overnight incubation with the primary anti-PGM5 antibody (diluted 1:5000 in 2% BSA/TBS-T; HPA067102, Sigma-Aldrich). Membranes were incubated with anti-rabbit secondary antibody (diluted 1:2500 in 2% BSA; W401, Promega, Madison WI, USA) for 1 h. Chemiluminescence detection system (GE Healthcare Life Sciences, Singapore) and ChemiDoc Imaging System (Bio-Rad) was used to visualise protein. β-actin acted as the loading control (diluted 1:2500 in 5% skim milk; 51255, Cell Signalling Technology, Danvers, MA, USA). Relative densitometry was measured using Image Lab software 6.0.1 (Bio-Rad).

Enzyme linked immunosorbent assay (ELISA)

Soluble fms-like tyrosine kinase-1 (sFLT-1) secretion was measured in conditioned culture media from cytotrophoblasts using the DuoSet Human VEGFR1/FLT-1 kit (R&D systems by Bioscience, Waterloo, Australia) according to manufacturer's instructions. Optical density was measured using a BioRad X-Mark microplate spectrophotometer and BioRad Microplate Manager 6 software.

Statistical analysis

In vitro experiments were performed in technical triplicates and repeated with $n \geq 3$ different patient samples. Data was tested for normality and statistically tested as appropriate. For two unpaired groups, an unpaired t-test (parametric) or Mann–Whitney test (non-parametric) test was used. For ≥ 3 groups, either one-way ANOVA (parametric) or a Kruskal Wallis test (non-parametric) was used. All data are expressed as mean ± SEM. p values < 0.05 were considered significant. Statistical analysis was performed using GraphPad Prism software 8 (GraphPad Software, Inc., San Diego, CA, USA).

Results

PGM5 is expressed in the human placenta throughout gestation

We first characterised *PGM5* expression in placental lysates throughout gestation. *PGM5* mRNA expression was detected in human placenta tissue collected at first trimester (7–11 weeks' gestation), preterm (24–30 weeks' gestation) and term (38–39 weeks' gestation). *PGM5* expression appeared to increase across gestation but this was not significant. *PGM5* expression was highly variable in first trimester tissue (Fig. 1).

PGM5 mRNA expression is downregulated in placenta collected from pregnancies complicated by preterm FGR and preeclampsia.

We next assessed whether *PGM5* expression was altered in placental tissue complicated by preeclampsia or FGR. *PGM5* expression was detectable in all samples assessed. *PGM5* expression was significantly decreased in

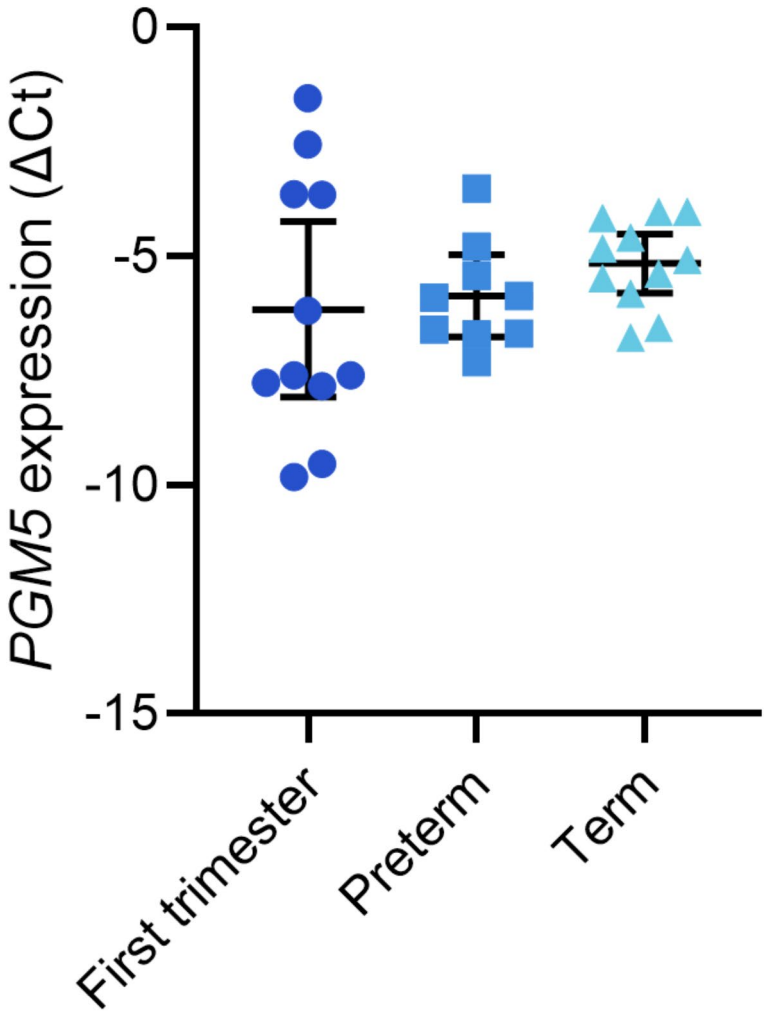


Fig. 1. *PGM5* expression in placental lysates across gestation. *PGM5* was expressed in placentas from first trimester, preterm, and term patients. A slight increase in *PGM5* expression can be observed over gestation however this trend is not significant. Data expressed as mean ± 95% confidence interval. First trimester (7–10 weeks' gestation) $n = 11$; early preterm (24–28 weeks' gestation) $n = 9$; term (38–39 weeks' gestation) $n = 11$. Individual symbols represent individual patients.

placental tissue from pregnancies complicated by preterm preeclampsia ($p = 0.0009$) and fetal growth restriction ($p = 0.0006$) (Fig. 2a), compared to gestation-matched preterm controls. Similarly, *PGM5* was decreased when preterm preeclampsia cases were split by pregnancies also complicated by FGR (Supplementary Fig. 1a). When split by fetal sex, *PGM5* expression was significantly decreased in cases of preeclampsia and FGR with a female fetus (Supplementary Fig. 1b). While placentas from pregnancies with a male fetus demonstrated decreased *PGM5* expression in pathology, the decrease was not statistically significant (likely due to lack of power in the control group; Supplementary Fig. 1c). Further analysis of the pathological cohort by simple linear regression revealed a positive association between *PGM5* expression and fetal birthweight (Fig. 2b, $R^2 = 0.234$, $p < 0.0001$).

PGM5 protein was variable in healthy and pathological (preeclampsia and FGR) preterm placenta

We next measured PGM5 protein in placental tissue lysates from pregnancies complicated by preterm preeclampsia and FGR. Densitometrical analysis of western blots revealed no change in PGM5 production in placental lysates complicated by preterm preeclampsia or fetal growth restriction relative to gestation-matched preterm controls (Fig. 3a and b; full blots in Supplementary Fig. 2). Notably, several placentas had undetectable, or very low PGM5 protein (Fig. 3a). The subset of placental tissue lacking PGM5 included samples from control and pathological (preeclampsia and FGR) pregnancies. Further review revealed there was no association with any available patient characteristics, including fetal sex (Supplementary Fig. 3a and b).

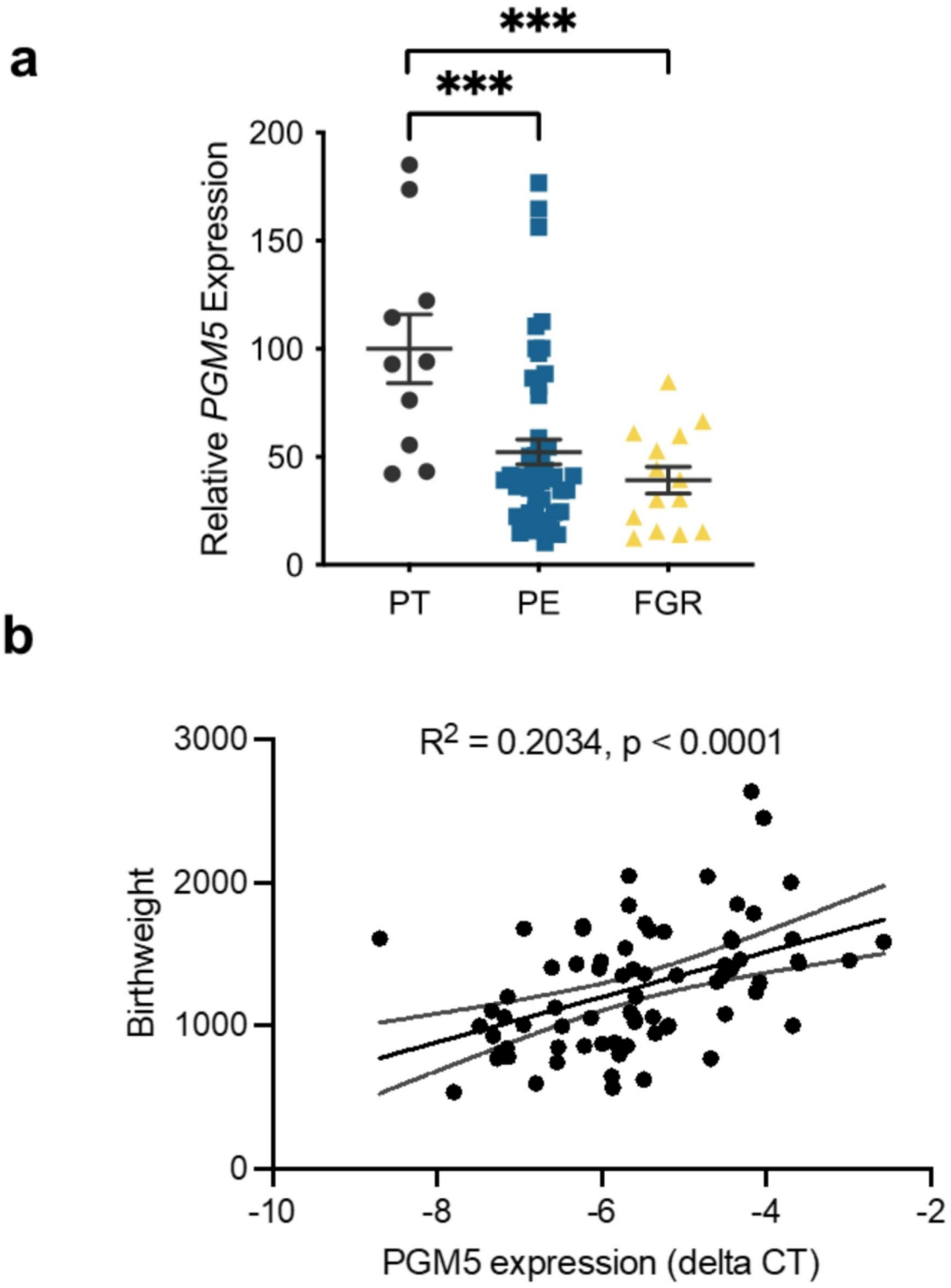


Fig. 2. *PGM5* expression in placental lysates complicated by preeclampsia (PE) and fetal growth restriction (FGR) relative to gestation-matched preterm controls (PT). (**a**) *PGM5* expression was significantly reduced in placentas from patients with early-onset preeclampsia, and patients with fetal growth restriction. (**b**) Placental *PGM5* expression was positively associated with birthweight. (**a**) Data expressed as fold change from control ± SEM. PT (black circles) $n = 10$, PE (blue squares) $n = 49$, FGR (yellow triangles) $n = 14$. (**b**) Simple linear regression was used to present a line of best fit ± 95% confidence interval of $n = 74$ patients. Individual symbols represent individual patients. $^{***}p < 0.001$.

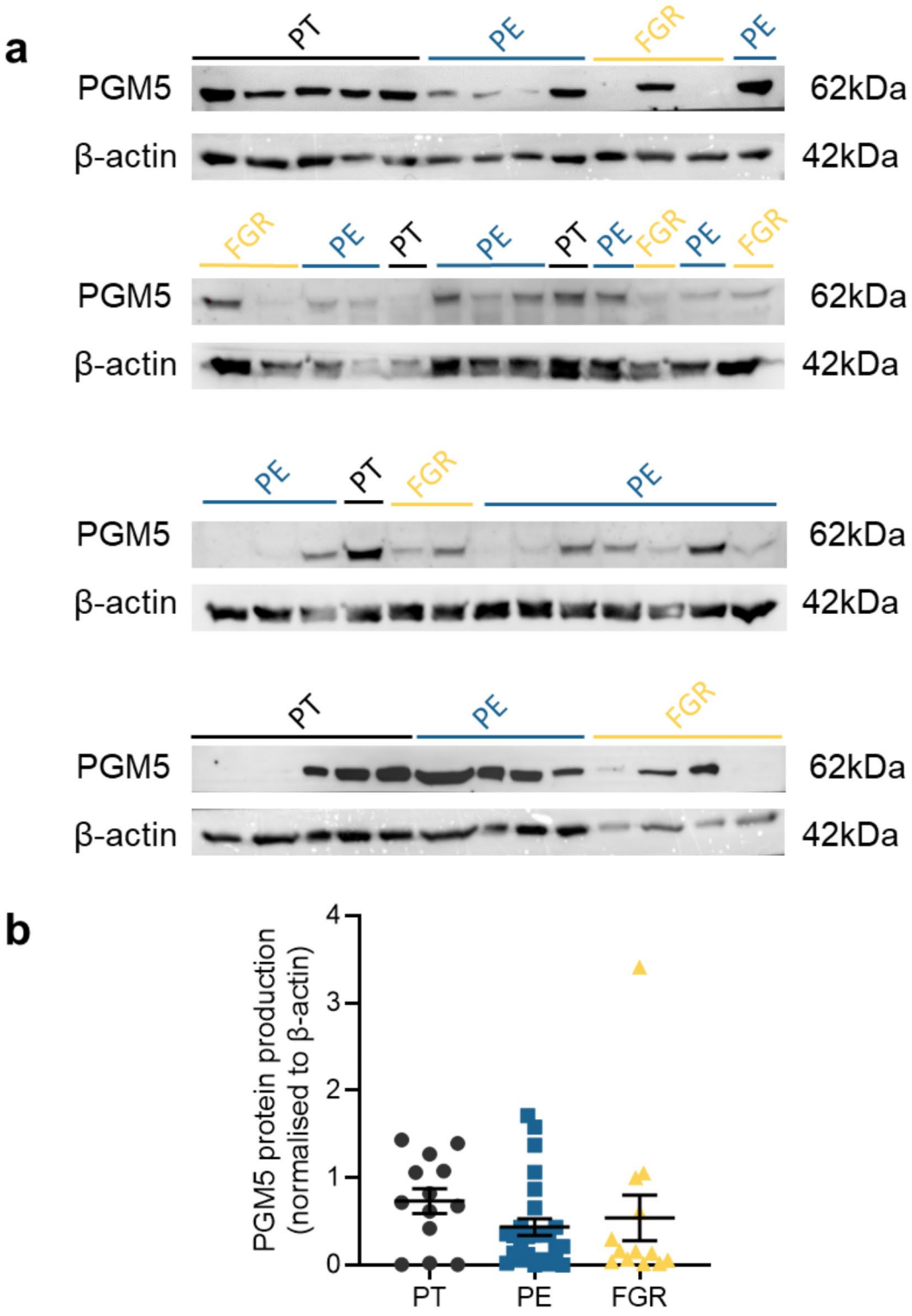


Fig. 3. PGM5 production in placental lysates from patients with early-onset preeclampsia (PE), fetal-growth restriction (FGR) and preterm controls (PT). (**a**) Representative western blots of PGM5 protein production. (**b**) Densitometric analysis of western blots. PGM5 protein expression was not altered in placental lysates from patients with PE or FGR relative to gestation matched controls. Several placental lysates from all three groups had undetectable or very low PGM5 expression. Data presented as mean ± SEM. β -actin acted as loading control. PT (black circles) $n = 13$, PE (blue squares) $n = 26$, FGR (yellow triangles) $n = 13$. Individual symbols represent individual patients.

PGM5 mRNA expression was significantly altered in placental explant tissue and cytotrophoblast under hypoxia

To investigate possible mechanisms underpinning the altered PGM5 levels in pathological placentas, *PGM5* was measured in placental cytotrophoblasts and explants exposed to hypoxic conditions to model conditions of placental dysfunction. *PGM5* mRNA expression was increased in primary cytotrophoblast cultures under hypoxic conditions, relative to normoxic control ($p < 0.0001$, Fig. 4a). PGM5 protein was not detectable in cytotrophoblast under either condition (data not shown). Similarly, *PGM5* expression was significantly increased in placental explants cultured under hypoxia relative to normoxic controls ($p = 0.0306$, Fig. 4b). However, densitometric analysis of western blots showed that PGM5 protein production was not altered in explants cultured under hypoxia (Fig. 4c and d; full blots in Supplementary Fig. 4).

Silencing *PGM5* under hypoxia increased MTS measured cell viability

To ascertain whether dysregulated PGM5 expression affects placental function, studies were conducted using short interfering (si)RNAs to silence *PGM5* expression in isolated primary cytotrophoblast. *PGM5* expression was reduced by approximately 70% under both normoxic ($p < 0.0001$, Fig. 5a) and hypoxic ($p < 0.0001$, Fig. 5b) culture conditions compared to negative (control) siRNA treatment, at each oxygen tension. MTS-assay as a measure of cell metabolic viability was unaltered when *PGM5* was silenced compared to negative siRNA control under normoxic conditions (Fig. 5c), but was significantly increased compared to negative siRNA control under hypoxic conditions, indicating increased metabolic activity/improved cell survival ($p = 0.0432$, Fig. 5d).

Silencing cytotrophoblast *PGM5* expression did not alter sFLT-1 secretion

sFLT-1 is a secreted anti-angiogenic factor, known to be elevated in preeclampsia[15]. To assess whether PGM5 has a role in altering sFLT-1, we first examined whether silencing *PGM5* alters expression of the sFLT-1 isoforms,

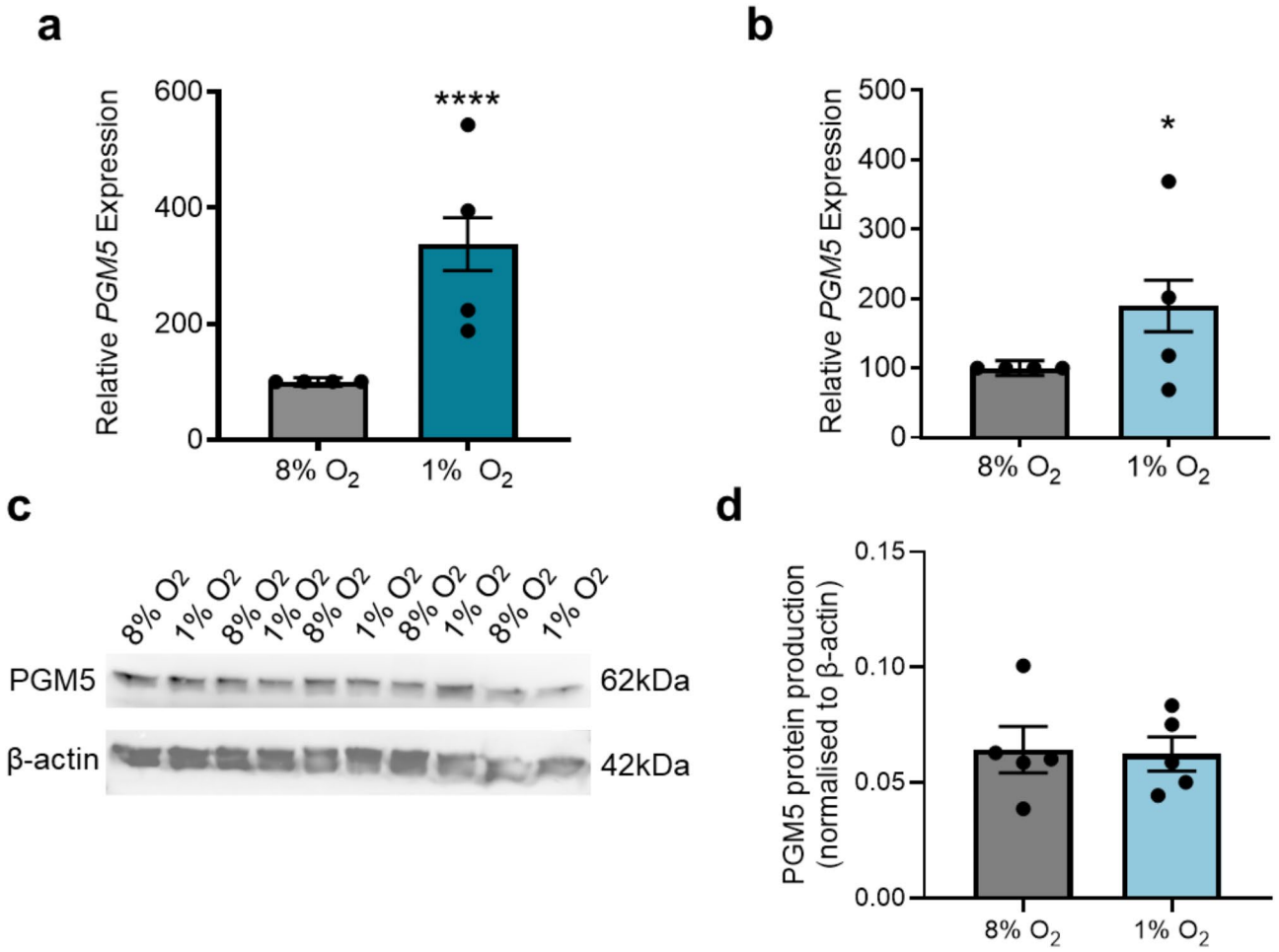


Fig. 4. PGM5 expression in placental cytotrophoblasts and explants exposed to hypoxia. *PGM5* expression was significantly increased in both cytotrophoblasts (**a**) and placental explants (**b**) exposed to hypoxia (1% oxygen) relative to normoxia (8% oxygen). However, densitometry analysis showed that PGM5 protein expression in placental explants was unaltered when exposed to hypoxia relative to normoxia conditions (**c**) and normalised to β -actin (d). Data expressed as fold change from control $\pm$ SEM. β -actin acted as loading control. $n = 4$–5 experimental replicates with individual symbols representing individual patients. *$p < 0.05$, ****$p < 0.0001$.

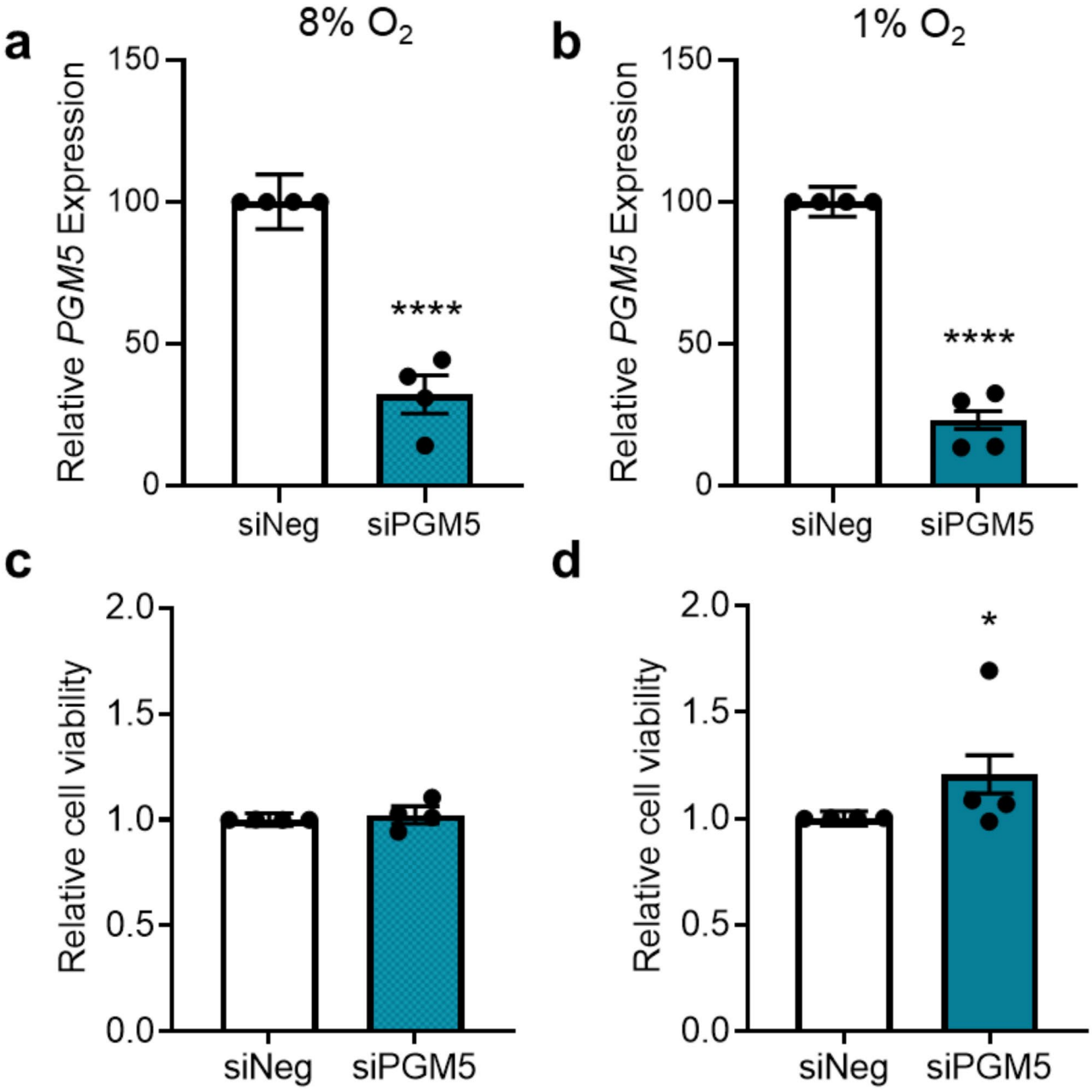


Fig. 5. Knockdown of PGM5 in primary trophoblasts and the effect on cell viability under normoxic and hypoxic conditions. Cytotrophoblast *PGM5* expression was significantly reduced following PGM5 siRNA (siPGM5) treatment under both normoxic (**a**) and hypoxic (**b**) conditions, relative to negative control siRNA (siNeg). Cell viability of PGM5 siRNA-treated cytotrophoblasts was unchanged under normoxic conditions (**c**) but increased under hypoxic conditions (**d**). Data expressed as fold change from control ± SEM. $n = 4$ experimental replicates, with each sample from a different patient. $^{*}p < 0.05$, $^{****}p < 0.0001$.

e15a and *i13*. No change in isoform expression was detected in PGM5-silenced cytotrophoblasts compared to negative siRNA control under normoxic conditions (Fig. 6a and b). However, under hypoxic conditions, silencing *PGM5* increased expression of *e15a* ($p = 0.0115$, Fig. 6c) but not *i13* (Fig. 6d) compared to negative siRNA control. Next, we assessed whether altered *sFLT-1* gene expression resulted in altered sFLT-1 protein secretion, however sFLT-1 secretion was not altered compared to respective negative siRNA control under either normoxic or hypoxic conditions (Fig. 6e and f).

Silencing *PGM5* upregulated apoptosis, growth and oxidative stress genes in cytotrophoblast cells

We next investigated whether silencing *PGM5* in cytotrophoblast led to altered expression of genes involved in key cellular pathways of apoptosis, growth and oxidative stress. Silencing trophoblast *PGM5* was associated

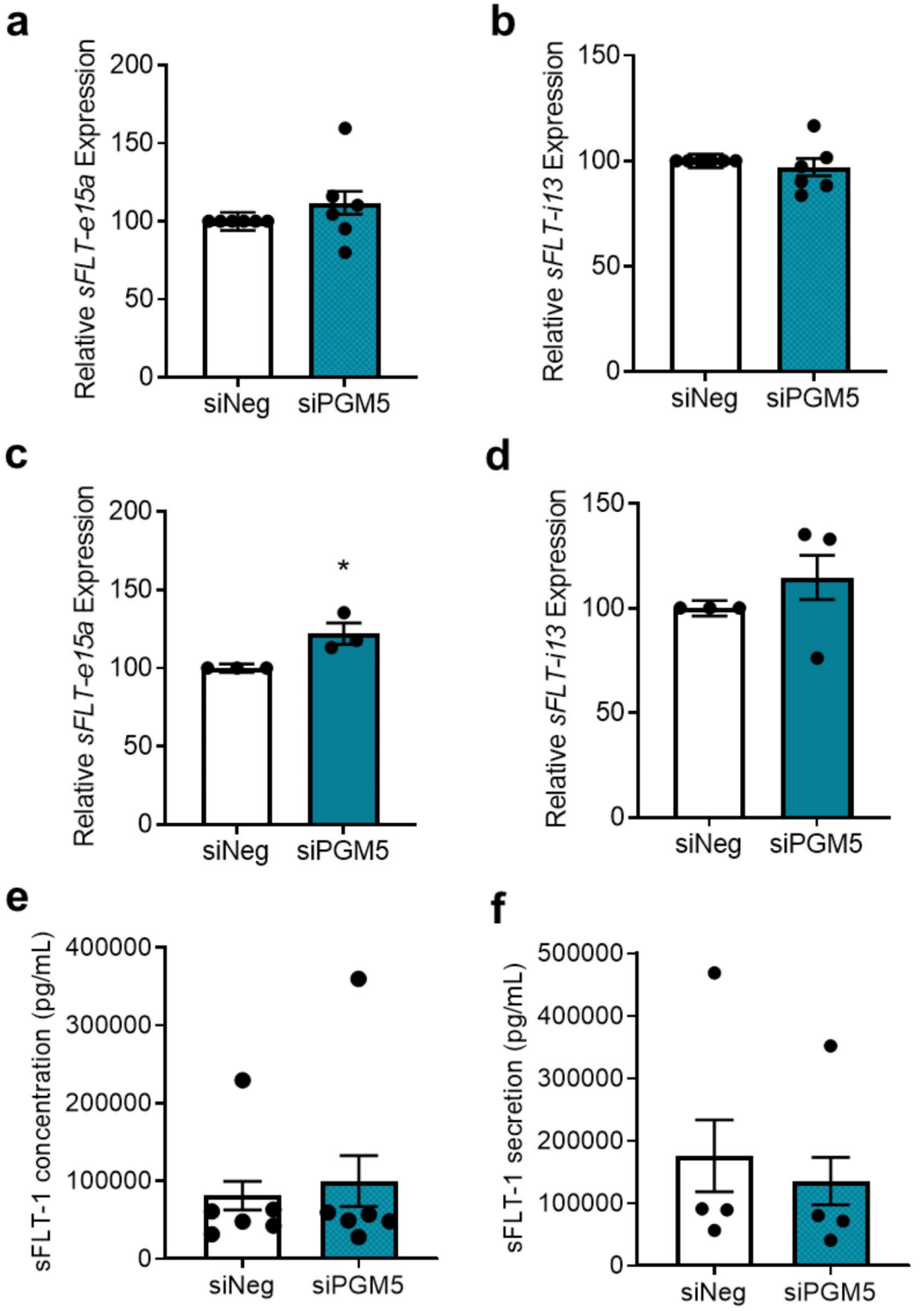


Fig. 6. *sFLT-1* expression in PGM5-silenced cytotrophoblasts (siPGM5). Silencing PGM5 did not alter the expression of sFLT-1 isoforms, *e15a* (**A**) and *i13* (**B**) under normoxic conditions relative to negative controls (siNeg). Following hypoxic exposure, PGM5-silenced cytotrophoblasts measured increased expression of the *sFLT-e15a* isoform (**C**) but not *i13* (**D**). Silencing *PGM5* in cytotrophoblasts did not alter protein production of sFLT-1 under normoxic (**E**) or hypoxic conditions (**F**). Data expressed as mean ± SEM. $n = 6$ experimental replicates for normoxic conditions and $n = 3–4$ for hypoxic conditions, with each sample from a different patient.

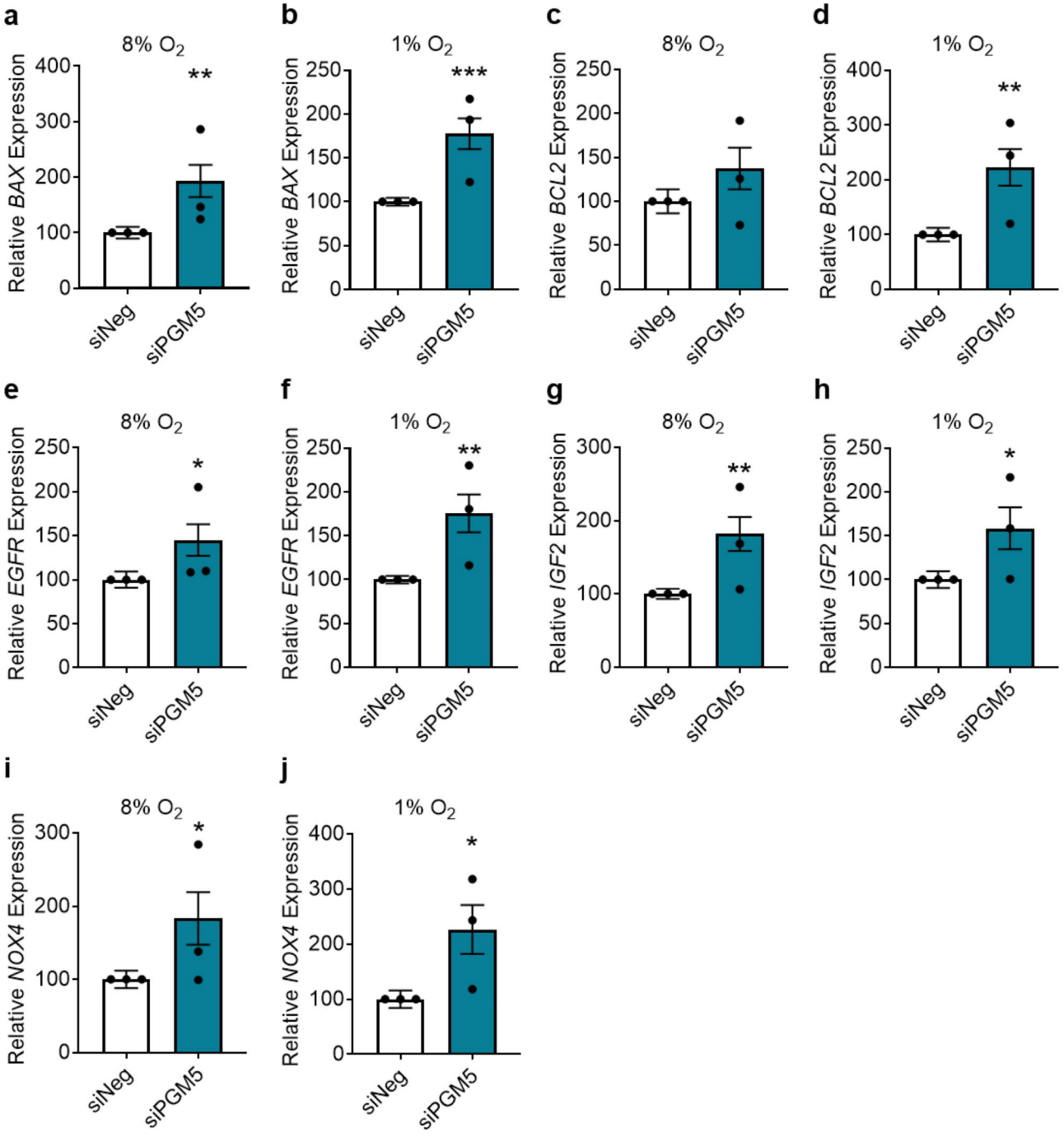


Fig. 7. The effect of silencing trophoblast PGM5 on genes associated with apoptosis, growth and oxidative stress. PGM5-silenced (siPGM5) trophoblasts measured elevated expression of pro-apoptotic gene, *BAX* under both normoxic (**A**) and hypoxic (**B**) conditions. The anti-apoptotic gene, *BCL2* was increased in PGM5-silenced trophoblasts under hypoxic (**D**) but not normoxic (**C**) conditions. Silencing *PGM5* in trophoblasts was associated with increased expression of growth-related genes, *EGFR* (normoxia, **E**; hypoxia, **F**) and *IGF2* (normoxia, **G**; hypoxia, **H**) and oxidative stress gene, NOX4 (normoxia, **I**; hypoxia, **J**) under both oxygen conditions. Data expressed as mean ± SEM. $N = 3$ experimental replicates with each sample from a different patient. $^{*}p < 0.05$, $^{**}p < 0.005$, $^{****}p < 0.0001$.

with an increase in expression of pro-apoptotic gene, *BAX* under both normoxic ($p = 0.0063$, Fig. 7a) and hypoxic conditions ($p = 0.0005$, Fig. 7b) compared to negative siRNA control (under matched oxygen tensions). Expression of *BCL2*, an anti-apoptotic gene, was not significantly altered in cytotrophoblast cells with *PGM5* knockdown under normoxic conditions (Fig. 7c), but was elevated where *PGM5* was silenced under hypoxic conditions ($p = 0.0033$, Fig. 7d) compared to respective negative siRNA control. Silencing *PGM5* in trophoblast

increased the expression of growth-related genes *EGFR* (normoxia $p = 0.0464$, Fig. 7e; hypoxia $p = 0.0040$, Fig. 7f) and *IGF2* (normoxia $p = 0.0028$, Fig. 7g; hypoxia $p = 0.0369$, Fig. 7h) compared to negative siRNA controls. *NOX4*, an oxidative stress gene, was also increased in trophoblast when *PGM5* was silenced under both normoxic ($p = 0.0274$, Fig. 7i) and hypoxic conditions ($p = 0.0159$, Fig. 7j), relative to matched control.

Discussion

In this study, we identified that *PGM5* is expressed in the human placenta throughout gestation and its expression is altered in pregnancies complicated by preeclampsia and fetal growth restriction, as well as models of placental dysfunction. We discovered that placental *PGM5* mRNA expression was decreased in placental tissue from pregnancies complicated by PE and/or FGR, which contrasted our overall observation of placental PGM5 protein production not being consistently altered in pathological and control tissue. In our knockdown assays, silencing *PGM5* expression in cytotrophoblasts drove upregulation in the expression of multiple genes associated with apoptosis, growth and oxidative stress. Collectively our findings suggest a role for PGM5 in human placental development, function and in disease.

PGM5 transcripts were highly elevated in the circulation of patients whose pregnancies are complicated with FGR and preeclampsia compared to healthy controls[5]. Though both conditions feature a dysfunctional placenta, in the current study, the placental expression did not reflect the highly elevated *PGM5* transcript levels detected in the blood, suggesting that the elevated *PGM5* transcripts in the circulation originate elsewhere. A possible source of these transcripts is tissues rich in smooth muscle, as studies have identified that this is where PGM5 is predominantly produced[9], as well as blood cells and reticulocytes which are the predominant contributors to circulating RNAs[16]. Understanding where these circulating transcripts originate, and whether these transcripts may have an effect within or on the placenta and other maternal organs is an area of interest for further investigation.

We discovered that *PGM5* is expressed in the human placenta in all trimesters throughout gestation. Few studies have investigated PGM5 in the human placenta previously, but one single cell sequencing study identified that *PGM5* expression in first trimester placenta was expressed in the fibroblasts rather than any trophoblast cell type[17]. This may be connected to one of the suggested roles of PGM5—its protein aciculin is believed to contribute to extracellular matrix and cellular structure, having first been identified in human uterine smooth muscle and fibroblasts[9]. Thus, lower *PGM5* expression could be associated with impacted aciculin production, and consequently disrupted placental cell matrix and tissue structure. Indeed, we found that there was an association between *PGM5* expression and birthweight in our cohort of placental samples from preterm pregnancies (both control and pathological). If this is indeed accurate, increased PGM5 may improve placental structure and integrity, and could be a potential avenue for treatment of placental insufficiency. However, this is speculative and further studies are needed.

Of note, we were surprised to see that certain placental samples, both pathological and control, did not have detectable PGM5 protein/aciculin production. This could mean that PGM5 is not essential for placental development (at least up to preterm gestation, as we did not assess protein in a large term cohort). An additional explanation could be that there is redundancy in its function, though further research would be needed to prove this. Redundancy is unlikely to be from other phosphoglucomutases from the same family, as PGM5 is the only one that has a structural role, with negligible phosphoglucomutase activity[9,18]. Alternatively, the absent PGM5 protein could reflect the heterogenous nature of the placenta and biopsies. Although the protocol for sample collection included tissue dissection from four sites across each placenta, it is possible that samples that were less vascularised, contained less stromal cells or had less adherens junctions may have less PGM5. Though previous studies have identified PGM5 protein localised to adherens junctions in human uterine smooth muscle[9], whether PGM5 has a similar distribution in the human placenta is unknown and warrants further investigation. Another view is that post-transcriptional or post-translational modifications could regulate the PGM5 protein production, but this also requires further investigation.

Single cell sequencing of first trimester placenta demonstrated *PGM5* expression in fibroblasts but not trophoblast cells[17]. Of note, we detected *PGM5* mRNA expression in our primary isolated cytotrophoblasts at term. This suggests that perhaps *PGM5* expression may be switched on later in gestation in cytotrophoblasts, or in culture. However *PGM5* expression may be transient, or temporally regulated. PGM5 protein was not detectable in cultured cytotrophoblast. The observations that *PGM5* has previously been shown to be produced by vasculature and fibroblasts lends support to its known roles in structural function, and suggests that PGM5 protein in whole placental tissue is unlikely to be derived from the cytotrophoblast. Furthermore, our study identified altered *PGM5* expression in our pathological placental tissue to that of our ex vivo and in vitro models of placental dysfunction. Whereby *PGM5* transcripts were reduced in placenta from pregnancies complicated by preterm preeclampsia and FGR compared to control. However, placental explant tissue and cytotrophoblasts cultured under hypoxia showed increased *PGM5* expression. This suggests that hypoxia is unlikely driving *PGM5* transcription differences in the FGR and preeclamptic placental tissue. However, hypoxia is just one aspect of the complex placental pathology underpinning FGR and preeclampsia.

Most literature on PGM5 comes from the cancer field, where loss of PGM5 is associated with poor prognosis in cases of colorectal, liver and prostate cancer – increasing tumor cell proliferation, invasion and migration[19–21]. As there are many similarities between a developing tumor and placenta, lower PGM5 might be considered beneficial to improve placental development. Trophoblast invasion and migration majorly occur in the early part of pregnancy, hence we suspect that this might not be as relevant at the gestation of our preterm/term samples – but could be important for the placental growth and proliferation that continues throughout gestation. Intriguingly, we found that silencing *PGM5* in cytotrophoblast increased the expression of many of the genes we assessed under hypoxia. This was seen whether or not the increase might be beneficial, for example growth genes *EGFR* and *IGF2* were increased, but so was the oxidative stress marker *NOX4*. This may suggest that *PGM5*

has a function that regulates many key cellular processes. Interestingly, these changes were also accompanied by increased cell viability, again suggesting that decreased PGM5 might be beneficial, and matches the increased viability (tumor progression) we see in the cancer field. More precise cellular viability measures are needed to validate this finding, and further investigation is required to untangle its function(s). However, we found that silencing cytotrophoblast *PGM5* did not increase the secretion of sFLT-1, suggesting that there is unlikely to be an association between the two. Hence PGM5 is unlikely to be a key driver of the elevated sFLT-1 secretion associated with preeclampsia pathogenesis.

A strength of our study is our use of well characterised primary human placental tissues from across gestation, from both healthy and pathological pregnancies with severe disease. However, a limitation of our first trimester samples is that we do not know whether these pregnancies would have continued on to be normal or pathological (though at termination, these pregnancies were progressing normally). Similarly, while gestation-matched preterm control placentas were used to control for confounding factors related to gestational age, the necessity to induce preterm birth implies that these pregnancies were not entirely 'normal'. Here we carefully excluded preterm placental tissue that was delivered due to infection, chorioamnionitis or hypertension related disorders. Another strength is our investigation of both protein and mRNA levels in the tissues, which highlighted distinct patterns of PGM5 regulation.

Conclusion

PGM5 codes for the aciculin protein that unlike its other phosphoglucomutase family members, has no enzymatic activity, rather functions as a structural extracellular and adherens junction molecule. However, little is known about PGM5 function in the placenta. Taken together, this study suggests that the presence of PGM5 in the placenta across gestation, and its ability to regulate many gene pathways implies that *PGM5* likely has a function in the placenta. However, the role and function of PGM5 in this specialised organ is still unclear. Further studies are needed to validate whether PGM5 plays a structural role in the placenta, and whether this might be manipulated to improve placental structure and mitigate dysfunction.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions
Conceptualisation, funding acquisition and supervision, N.J.H.; Methodology, N.d.A., S.B., N.K.B. and N.J.H.; Formal analysis, N.d.A., S.B. and N.J.H.; Investigation, N.d.A., S.B. and N.K.B.; Resources, N.J.H., S.T., T.J.K.-L. and L.H.; Sample collection and characterization, N.P., L.H. and S.T.; Writing (original draft preparation), N.d.A., L.B. and N.J.H.; Visualization, N.d.A., L.B. and S.B. Writing (review and editing), all authors. All authors have read and agreed to the published version of the manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

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