



OPEN

Hyperglycaemia up-regulates placental growth factor (PlGF) expression and secretion in endothelial cells via suppression of PI3 kinase-Akt signalling and activation of FOXO1

Samir Sissaoui^{1,5}, Stuart Egginton², Ling Ting¹, Asif Ahmed^{3,4} & Peter W. Hewett¹✉

Placenta growth factor (PlGF) is a pro-inflammatory angiogenic mediator that promotes many pathologies including diabetic complications and atherosclerosis. Widespread endothelial dysfunction precedes the onset of these conditions. As very little is known of the mechanism(s) controlling PlGF expression in pathology we investigated the role of hyperglycaemia in the regulation of PlGF production in endothelial cells. Hyperglycaemia stimulated PlGF secretion in cultured primary endothelial cells, which was suppressed by IGF-1-mediated PI3K/Akt activation. Inhibition of PI3K activity resulted in significant PlGF mRNA up-regulation and protein secretion. Similarly, loss or inhibition of Akt activity significantly increased basal PlGF expression and prevented any further PlGF secretion in hyperglycaemia. Conversely, constitutive Akt activation blocked PlGF secretion irrespective of upstream PI3K activity demonstrating that Akt is a central regulator of PlGF expression. Knock-down of the Forkhead box O-1 (FOXO1) transcription factor, which is negatively regulated by Akt, suppressed both basal and hyperglycaemia-induced PlGF secretion, whilst FOXO1 gain-of-function up-regulated PlGF in vitro and in vivo. FOXO1 association to a FOXO binding sequence identified in the PlGF promoter also increased in hyperglycaemia. This study identifies the PI3K/Akt/FOXO1 signalling axis as a key regulator of PlGF expression and unifying pathway by which PlGF may contribute to common disorders characterised by endothelial dysfunction, providing a target for therapy.

Diabetes mellitus is associated with both central and peripheral vasculopathies, notably diabetic retinopathy¹. Patients also have an increased incidence of cardiovascular disease (CVD), susceptibility to associated complications such as plaque rupture and atherothrombosis, and approximately 75% die from vascular-related disease^{1,2}. Intensive management of blood glucose significantly reduces the risk of microvascular complications³. Excessive superoxide anion generation in the mitochondria was proposed as the common mechanism underlying vascular injury in response to hyperglycaemia, although other factors such as insulin resistance, diabetic dyslipidemia, and advanced glycation end products contribute in type-2 diabetes^{2,4,5}. Alterations in endothelial function precede the onset of diabetic complications, and are characterised by reduced endothelial nitric oxide (NO) synthase activity, elevated levels of inflammatory and coagulation markers, and increased endothelial cell apoptosis⁶.

The phosphoinositide-3'-kinase (PI3K)/Akt (PKB) pathway is important for both mature endothelial cell and progenitor cell function^{7,8} and its activity is reduced in both type-1 and type-2 diabetes mellitus⁶. Several mechanisms may contribute to the down-regulation of PI3K signalling in type-2 diabetes including the up-regulation

¹Institute of Cardiovascular Sciences, College of Medical and Dental Sciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK. ²Multidisciplinary Cardiovascular Research Centre, University of Leeds, Leeds LS2 9JT, UK. ³MyrZyme Therapeutics Ltd, Faraday Wharf, Innovation Birmingham Campus, Holt Street, Birmingham B4 4BB, UK. ⁴School of Health Sciences, University of Southampton, Southampton SO17 1BJ, UK. ⁵Present address: Arima Genomics, 6404 Nancy Ridge Drive, San Diego, CA 92121, USA. ✉email: p.w.hewett@bham.ac.uk

of TBR3, a CDC25 binding protein homolog, which directly inhibits Akt activity⁹, and induction of the PI3K antagonist PTEN (phosphatase and tensin and homologue deleted on chromosome 10)¹⁰. Alternatively, there may be reduced levels of ligands and/or receptor activity coupled to this pathway.

Placenta growth factor (PlGF) is a member of the vascular endothelial growth factor (VEGF) family that mediate vascular development and homeostasis. Like VEGF, PlGF is a pleiotropic cytokine that promotes angiogenesis, vascular remodelling, cell survival and inflammation¹¹. PlGF acts via the VEGF receptor-1 (VEGFR-1) and neuropilin accessory receptors, but leads to the phosphorylation of different VEGFR-1 tyrosine residues and gene expression to those activated by VEGF-A¹². The expression of PlGF is low, or absent, in most healthy adult tissues and its activity is reported to be confined to pathological situations such as diabetes, atherosclerosis, arthritis and cancer making it an attractive target for therapy¹¹.

Our laboratory reported high vitreous PlGF levels in patients with proliferative diabetic retinopathy¹³, which was confirmed by others^{14,15}. The intravitreal or systemic delivery of PlGF in rodents disrupts retinal barrier function and increases vascular leakage in a VEGFR-1-dependent manner, mimicking diabetic retinopathy^{15,16}. In addition, administration of soluble VEGFR-1 receptor (sFlt-1), which sequesters both PlGF and VEGF, prevents diabetic retinopathy in a spontaneous rat model^{11,17}. Furthermore, delivery of an anti-PlGF antibody in a mouse diabetic retinopathy model reduced vascular leakage, inflammation and fibrosis¹⁸. Elevated circulating PlGF levels are associated with vascular inflammation and adverse outcome in patients with acute coronary syndrome¹⁹, severity of metabolic syndrome²⁰, development of type-2 diabetes²¹ and childhood obesity²². Increased PlGF expression in atherosclerotic plaques correlate with plaque instability, inflammation and intimal angiogenesis²³. In atherosclerosis-prone apolipoprotein E deficient (ApoE^{-/-}) mice, PlGF is up-regulated in early lesions and loss of PlGF reduces plaque size and macrophage content^{24,25}. Interestingly, PlGF null mice also have a lower fat content when maintained on a high-fat diet²⁶. However, the mechanism(s) by which PlGF expression is up-regulated in these pathologies remain unknown. In this study we show that manipulation of glucose concentration, PI3K or Akt activity leads to consistent changes in endothelial cell PlGF expression and release, which are dependent on FOXO1 transcription factor activity. The identification of the involvement of the PI3K/Akt/FOXO1 pathway in the control of PlGF expression may have important implications for endothelial homeostasis and dysfunction, and therefore represent a target for therapy in vascular disorders.

Results

PlGF expression is modulated by hyperglycaemia in endothelial cells. Elevated circulating PlGF has recently been identified in insulin resistance, metabolic syndrome, vascular inflammation and is an independent marker of the future development of type-II diabetes^{19–22}. To determine whether hyperglycaemia up-regulates the expression of PlGF, we incubated confluent primary human endothelial cells in medium containing 30 mM D-glucose for 24 h and measured the level of PlGF released into the medium by ELISA. Hyperglycaemia significantly up-regulated PlGF release from HUVEC (Fig. 1A) and HAEC (Fig. S1) compared with cells maintained under normal glucose levels (5 mM D-glucose) or osmolarity controls (25 mM L-glucose + 5 mM D-glucose). As hyperglycaemia is reported to suppress PI3K signalling in many cell types, including endothelial cells²⁶ we examined the effect of the established PI3K activators, IGF-1 and insulin on PlGF expression. The basal level of PlGF produced by endothelial cells was reduced in a concentration-dependent manner by IGF-1 (Fig. 1B) and insulin (1 mM) led to a 31% decrease in PlGF secretion (data not shown), indicating that PlGF release is negatively regulated by PI3K pathway activity in endothelium. Furthermore, addition of IGF-1 (10 ng/ml) significantly reduced the level of PlGF release in endothelial cells grown under both hyperglycaemic and normoglycaemic conditions (Fig. 1C and Fig. S1). The inhibitory effect of hyperglycaemia on PI3K activity in HUVEC and its modulation with IGF-1 (10 ng/ml) were demonstrated by Western blotting for phosphorylation of Akt at Serine 473 (pAkt^{Ser473}). The level of activity (pAkt^{Ser473}) was found to be decreased by hyperglycaemia under both basal conditions and following IGF-1 stimulation in comparison with cells in normoglycaemia (Fig. 1D).

Up-regulation of PlGF expression was reported to be dependent on endogenous VEGF in bovine retinal endothelial cells²⁷. To determine whether endogenous VEGF was involved in the observed up-regulation of PlGF in hyperglycaemia, VEGF was knocked-down in HUVEC prior to exposure to 30 mM D-glucose (Fig. S2). Surprisingly, knock-down of endogenous VEGF expression was found to promote PlGF mRNA expression and secretion under control conditions (Fig. S2A–C) and in the presence of hyperglycaemia indicating that PlGF production in hyperglycaemia is not secondary to the induction of endogenous VEGF (Fig. S2D). This also consistent with an observed decrease in Akt phosphorylation 24 h after the knock-down of endogenous VEGF (data not shown).

PI3K signalling negatively regulates PlGF expression in endothelial cells. To establish the involvement of the PI3K pathway in controlling PlGF expression, HUVEC were incubated with the PI3K inhibitor, LY294002, and PlGF expression and release examined by real-time qPCR and ELISA. Blockade of PI3K resulted in a significant increase in both PlGF mRNA and protein release (Fig. 2A,B). In addition, the use of recombinant adenoviruses encoding the PI3K antagonist, PTEN, produced a similar increase in PlGF release from HUVEC, whilst cells infected with a catalytically inactive PTEN mutant (PTENC/s) were not affected (Fig. 2C). Western blotting was performed to confirm that LY294002 treatment and PTEN over-expression led to a decrease in the activation of the downstream PI3K effector, Akt (Fig. 2D,F). This was demonstrated by the greatly reduced levels of phosphorylation of Akt serine 473 (pAkt^{Ser473}). Furthermore, no further increase in PlGF release was observed from HUVEC cultured in medium containing 30 mM D-glucose following pre-treatment with LY294002, indicating that hyperglycaemia stimulates PlGF expression via its inhibitory action on the PI3K pathway (Fig. 2E).

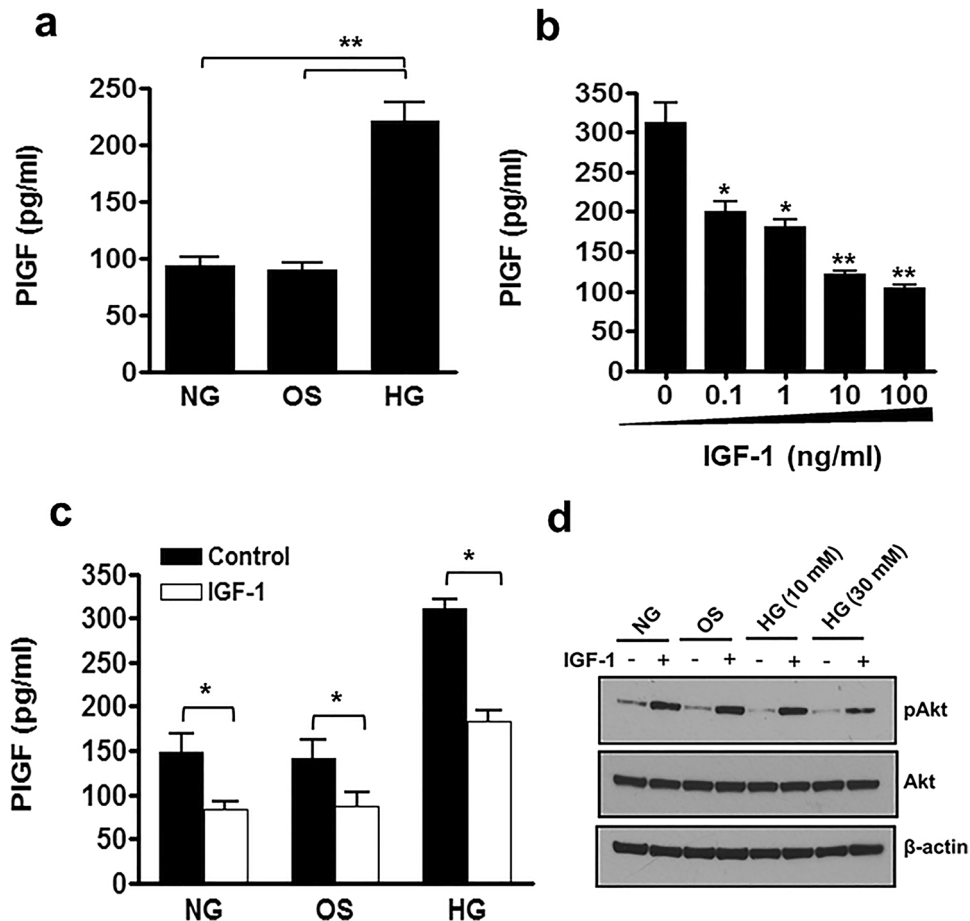


Figure 1. Hyperglycaemia suppresses Akt activity and promotes PIGF secretion in endothelial cells. (a) Confluent endothelial cells cultured for 24 h in either normoglycaemia (NG = 5 mM D-glucose), hyperglycaemia (HG = 30 mM D-glucose) or osmolarity control (OS = 5 mM D-glucose + 25 mM L-glucose) medium, and the level of PIGF secretion measured by ELISA. (b) Confluent HUVEC stimulated with increasing concentrations of IGF-1 in medium containing 10% FCS for 24 h and PIGF secretion measured by ELISA. (c) HUVEC were cultured in medium containing either NG, HG or OS control in the presence of IGF-1 (10 ng/ml) for 24 h and PIGF secretion quantified by ELISA. (d) Confluent HUVEC were maintained in NG, HG or OS control conditions and then stimulated with IGF-1 (10 ng/ml) or vehicle for 10 min and cell lysates Western blotted for phospho-Akt^{Ser473} (pAkt^{Ser473}), total Akt and β-actin. The unedited blot images are presented in Supplementary Figure S5. Results are the mean ± SEM of 3 or more independent experiments; Student's t-test *P < 0.05, **P < 0.01.

Akt is a negative regulator of PIGF expression in endothelial cells. To determine whether PI3K acts via Akt to regulate PIGF expression, endothelial cells were infected with adenoviruses encoding either dominant-negative (dn-Akt), or constitutively active myristolated (myr-Akt) mutants of Akt. Following infection of HUVEC with dn-Akt adenoviruses (see Fig. S3), the levels of PIGF mRNA and protein released into the culture medium increased significantly compared with cells treated with control virus encoding β-galactosidase (β-gal) (Fig. 3A,B). To further establish the central role of Akt in the regulation of PIGF expression, HUVEC were infected with different combinations of either PTEN or β-gal with PTEN, myr-Akt or β-gal adenoviruses to maintain equal viral load. Over-activation of Akt dramatically reduced the increase in PIGF release stimulated by PTEN-mediated blockade of the PI3K pathway, demonstrating that Akt is the key downstream effector regulating PIGF expression (Fig. 3C). Moreover, the involvement of Akt activity in the hyperglycaemic induction of PIGF was confirmed by the over-expression of myr-Akt, which inhibited hyperglycaemia-induced PIGF release (Fig. 3D).

Akt-1 is the predominant isozyme of Akt in the vasculature and is involved with the regulation of several IGF-1 target genes and implicated in various pathologies⁶. The siRNA-mediated knock-down of Akt-1 in HUVEC (Fig. 3E) significantly increased PIGF secretion into the culture medium (Fig. 3F). In contrast to normoglycaemia and the osmolarity control, the loss of Akt1 did not result in any further increase of PIGF secretion compared with control siRNA treated cells under hyperglycaemic conditions (Fig. 3G). Collectively, our findings show that Akt is a negative regulator of PIGF production in endothelial cells.

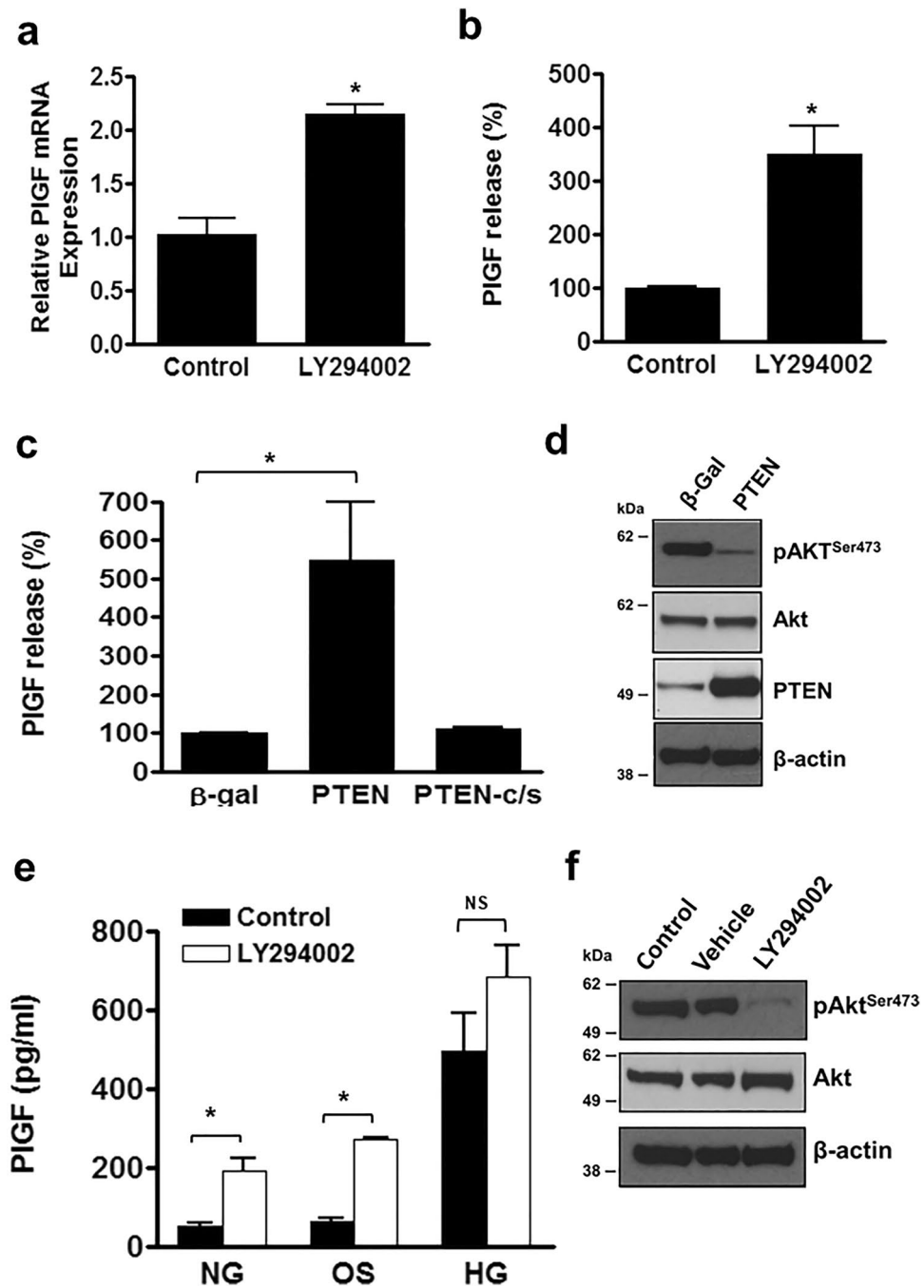


Figure 2. Inhibition of the PI3K pathway promotes PIGF expression and secretion. Confluent HUVEC pre-treated with LY294002 (20 μ M) and incubated for (a) 8 h or (b) 24 h and PIGF mRNA quantified by qPCR and protein secretion by ELISA. (c) HUVEC were infected overnight with adenoviruses encoding PTEN, mutant PTEN (PTEN-c/s), or β -galactosidase (β -gal). Following 24-h incubation (c) PIGF levels in the culture medium were quantified by ELISA. (d) Western blots confirming inhibition of Akt activity in HUVEC following Ad-PTEN infection. (e) HUVEC treated with LY294002 or the vehicle and incubated for 24 h in normoglycaemia (NG = 5 mM D-glucose), hyperglycaemia (HG = 30 mM D-glucose), or osmolarity control (OS = 5 mM D-glucose + 25 mM L-glucose), and PIGF measured in supernatants by ELISA. (f) Representative Western blots of the HUVEC lysates following treatment of HUVEC with LY294002 or the vehicle for one hour showing pAkt^{Ser473}, total Akt with β -actin as a loading control. The unedited blot images are presented in Supplementary Fig. S5. Results are the mean \pm SEM of 3 or more experiments ($n > 6$), Student t-test; * $P < 0.05$, ** $P < 0.01$.

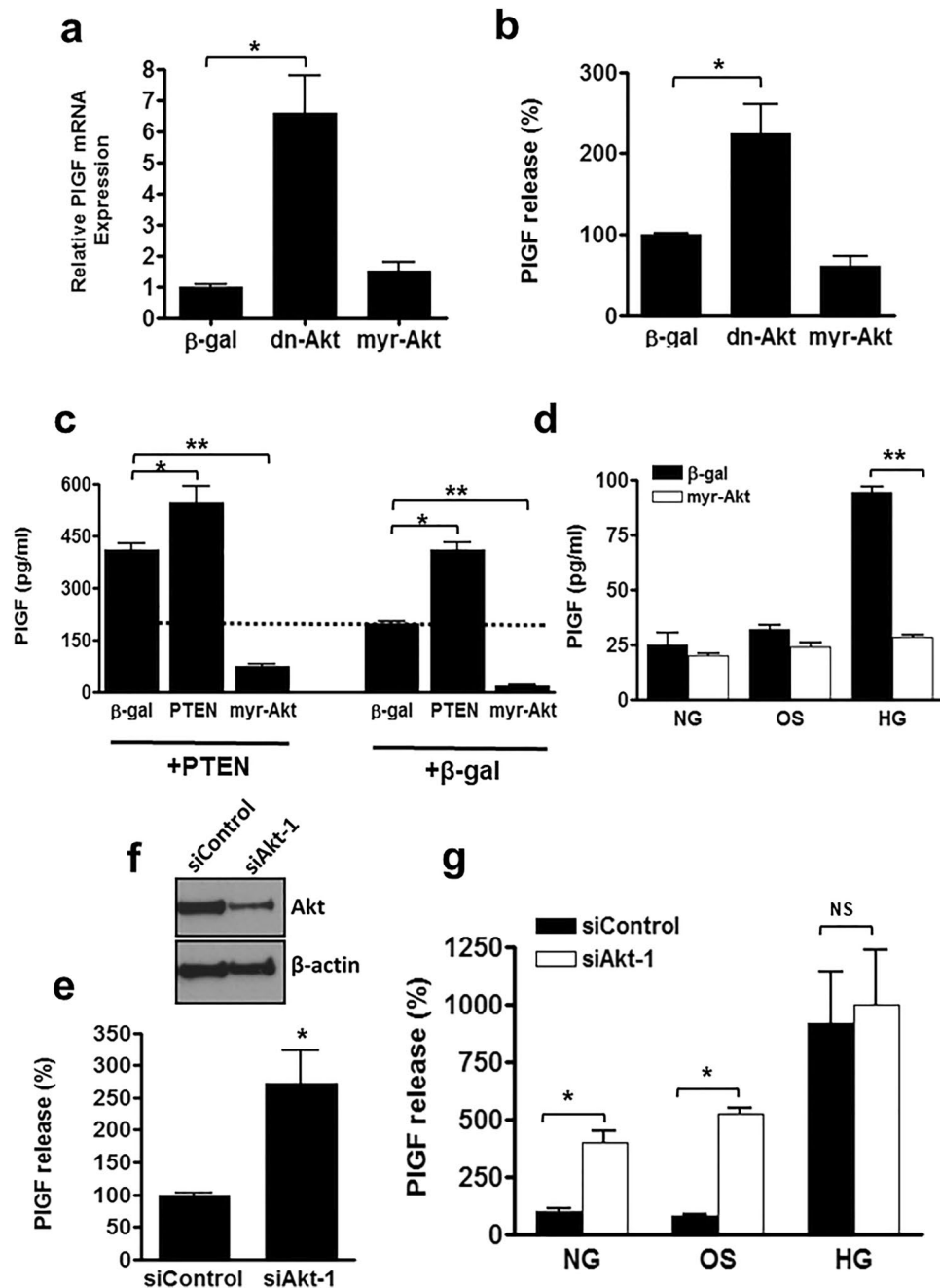


Figure 3. Inhibition of Akt activity increases PIGF production. HUVEC were transduced with adenoviruses encoding dominant-negative Akt (dn-Akt), constitutively active Akt (myr-Akt) and β-galactosidase (β-gal). Following (a) 8 h and (b) 24-h incubation PIGF expression and secretion was quantified by qPCR and ELISA. (c) HUVEC were co-infected with combinations of PTEN, myr-Akt and β-gal (control) adenoviruses overnight. After 24 h PIGF secretion was quantified by ELISA. (d) HUVEC were transduced with myr-Akt or β-gal and incubated in normoglycaemia (NG = 5 mM D-glucose), hyperglycaemia (HG = 30 mM D-glucose) or osmolarity control (OS = 5 mM D-glucose + 25 mM L-glucose), and PIGF release into the culture medium measured by ELISA. (e–g) HUVEC were electroporated with siRNA targeted to Akt-1 (siAkt) or control siRNA (siControl). (e) PIGF measured after 24 h in cell supernatants and (f) the cell lysates Western blotted for Akt and β-actin (unedited blot images in Supplementary Figure S5). (g) Cells were incubated in NG, HG or OS for 24 h and PIGF release measured by ELISA. Results are the mean ± SEM of 3 or more experiments (n ≥ 6); Student's t-test, *P < 0.05, **P < 0.01.

FOXO1 promotes PIGF expression in endothelial cells following hyperglycaemia. The forkhead box O (FOXO) transcription factors are inversely regulated by Akt activation through phosphorylation, which leads to inhibition of DNA binding and their exclusion from the nucleus²⁸. FOXO1 is the predominant FOXO expressed in the vasculature, adipose tissue, liver and pancreas and is closely linked to glucose metabolism and the regulation of insulin/IGF-1-sensitive genes²⁸. Consistent with our findings of reduced Akt activity (Fig. 1D), the level of FOXO1 Serine 256 phosphorylation (pFOXO1^{Ser256}) decreased in these cells following exposure to hyperglycaemia indicating an increase of FOXO1 activity (Fig. 4A). Treatment of HUVEC with IGF-1 led to large increase FOXO1 phosphorylation/inhibition in normoglycaemia and the osmolarity control. There was a marked reduction in the level of pFOXO1^{Ser256} in cells incubated with IGF-1 in hyperglycaemia (30 mM glucose) (Fig. 4A). These findings are consistent with the reduced level of Akt phosphorylation under these conditions (Fig. 1D). Similar effects on FOXO1 phosphorylation were also observed following infection of HUVEC with adenovirus expressing myr-Akt (Fig. S3). FOXO1 knock-down in HUVEC resulted in a significant decrease in PIGF mRNA expression and protein release demonstrating the importance of FOXO1 activity in basal PIGF release (Fig. 4B and Fig. S4). In contrast, knock-down of the closely related FOXO3A transcription factor did not affect PIGF expression or release (*see* Fig. S4). To confirm the requirement of FOXO1 in the hyperglycaemic induction of PIGF, HUVEC were incubated in medium containing 30 mM glucose following siRNA-mediated FOXO1 knock-down, which abrogated hyperglycaemia-induced PIGF up-regulation (Fig. 4C). Consistent with these findings, the adenoviral-mediated over-expression of constitutively active FOXO1 (caFoxO1) induced a fourfold increase in PIGF production compared with the β -gal control (Fig. 4D). No reduction in PIGF levels were observed following IGF-1 stimulation of caFOXO1 transduced HUVEC in contrast to the significantly reduced PIGF release in the β -gal controls (Fig. 4D).

In silico analysis of the human PIGF gene sequence identified a FOXO consensus DNA binding sequence (containing the core 5'-AAACAA-3' motif) between -3414 bp and -3423 bp upstream of the PIGF transcriptional start site. Enrichment of FOXO1 binding to this site was investigated using ChIP assays performed with qPCR amplification a 118 bp region flanking the putative FOXO1 binding site relative to a negative control DNA region (NegC). As an additional control, FOXO1 enrichment was also examined at a region flanking the TATAA box proximal to the PIGF transcriptional start site, and compared with the degree of DNA polymerase II (Pol II) enrichment at both sites. Endogenous FOXO1 binding was found to be enriched at the FOXO1 site in the PIGF promoter, which increased significantly (~fourfold) in HUVEC cultured under hyperglycaemic conditions (Fig. 4E). As expected there was no enrichment of Pol II binding at this site irrespective of glycaemic conditions (Fig. 4E). Conversely, as expected significant enrichment of Pol II was detected proximal to the PIGF transcriptional start site under normoglycaemic conditions which increased (sixfold) in hyperglycaemia, consistent with the up-regulation of PIGF expression in hyperglycaemia (Fig. 4F). As expected there was no enrichment of FOXO1 at the transcriptional start site (Fig. 4F), or Pol II at the FOXO1 binding site (Fig. 4E) further demonstrating specificity of the chromatin precipitation. Collectively, these data demonstrate that FOXO1 activity is required for the direct induction of PIGF expression in hyperglycaemia.

FOXO1 promotes PIGF expression in vivo. To determine whether increased FOXO1 activity can regulate PIGF expression in vivo, recombinant adenoviruses encoding either caFoxO1 (Ad-caFoxO1) or control empty virus (Ad-CMV) were delivered systemically to FVB/N mice. After 48 h the mice were culled and the levels of FoxO1 and Plgf mRNA in the liver and PIGF protein in plasma, quantified by qPCR and ELISA respectively (Fig. 5). The level of FoxO1 mRNA was found to be increased the livers of the Ad-caFOXO1 group compared with the Ad-CMV control group confirming adenoviral transduction (Fig. 5A). Figure 5B, shows that PIGF mRNA expression was significantly greater in the livers of mice receiving the Ad-caFoxO1 compared with Ad-CMV controls. Furthermore, circulating levels of PIGF in mice treated with caFoxO1 also increased significantly compared with the Ad-CMV control group (Fig. 5C).

Discussion

The PI3K/Akt pathway is an important regulator of vascular homeostasis and loss of Akt activity results in endothelial dysfunction, which is a characteristic of diabetes, atherosclerosis, and the metabolic syndrome. In this study, we demonstrate that hyperglycaemia directly up-regulates PIGF by suppression of PI3K/Akt signalling resulting in an increase in FOXO1 activity and binding to the PIGF promoter in endothelial cells, and increased FOXO1 activity promotes PIGF expression in vivo. Akt phosphorylation is reported to be reduced by 50% in the internal mammary arteries of type-2 diabetic patients²⁷. Similarly, a loss of Akt activity is also reported in models of diabetes^{26,29} and suppression of the PI3K antagonist PTEN restores Akt activity improving insulin sensitivity in mouse models of diabetes¹⁰. Hyperglycaemia was reported previously to inhibit the PI3K/Akt pathway in vitro in rat retinal endothelial cells²⁶. This is consistent with our findings of reduced Akt phosphorylation in HUVEC incubated under hyperglycaemia, both under basal conditions and in the presence of IGF-1, and corresponding changes in FOXO1 phosphorylation/activity in these cells. This resulted in increased FOXO1 binding to the PIGF promoter in HUVEC incubated under hyperglycaemic conditions. These findings are supported by a concomitant increase in Pol II enrichment in the region proximal to the PIGF transcriptional start site. Our data also show that Akt is the major downstream PI3K effector involved in PIGF production. The role of PI3K in PIGF expression was demonstrated by direct manipulation of the PI3K/Akt pathway using pharmacological inhibitors, recombinant IGF-1, or over-expression of wild-type and mutant signalling proteins in endothelial cells. Over-expression of myr-Akt, in the presence of PTEN-mediated inhibition of PI3K, or hyperglycaemia, markedly attenuated PIGF release. Knock-down of Akt1 in endothelial cells produced a similar increase in PIGF production to the general inhibition of Akt activity following over-expression of a dominant-negative Akt mutant, highlighting the importance of the Akt1 isozyme in regulating PIGF production. Akt1 is the predominant form

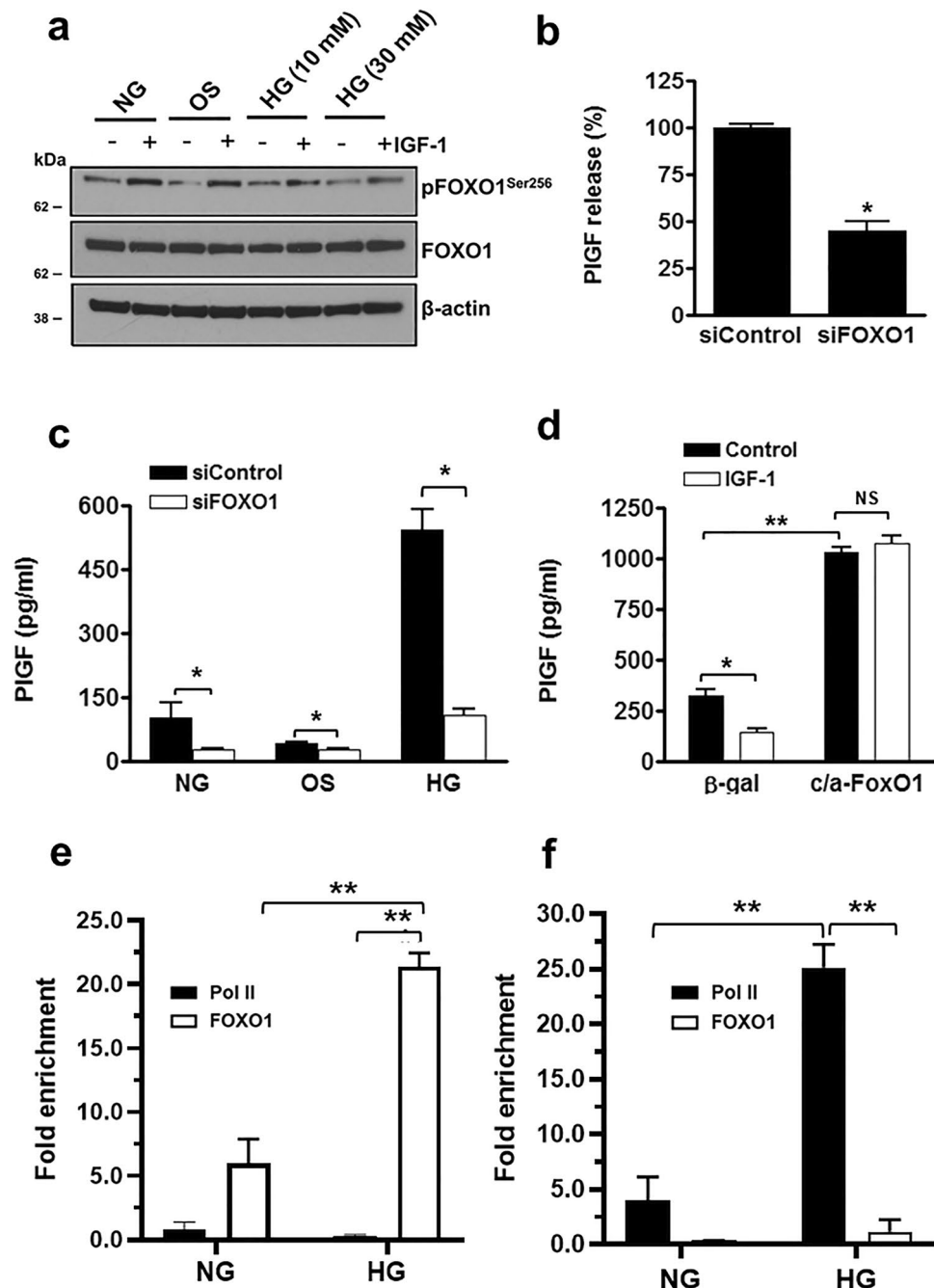


Figure 4. Increased FOXO1 activity induces PIGF expression and release. **(a)** Confluent HUVEC were maintained in normoglycaemia (NG = 5 mM D-glucose), hyperglycaemia (HG = 10, or 30 mM D-glucose) or osmolarity control medium (OS = 5 mM D-glucose + 25 mM L-glucose) and then stimulated with IGF-1 (10 ng/ml) for 10 min, and cell lysates Western blotted for phospho-FOXO1^{Ser256}, FOXO1 and β-actin (reused from Fig. 1d), with unedited blot images in Supplementary Fig. S5. **(b)** HUVEC were electroporated with FOXO1 (siFOXO1) or control (siControl) siRNAs PIGF secretion into the culture medium quantified by ELISA. **(c)** HUVEC were cultured in NG, HG, or OS for 24 h and stimulated with IGF-1 (10 ng/ml) for 10 min. **(d)** HUVEC infected with ca-FoxO1 and Ad-CMV (control) adenoviruses overnight, then incubated with IGF-1 (10 ng/ml) and PIGF quantified in cell supernatants by ELISA. Quantitative PCR detection of FOXO1 and Pol II enrichment to **(e)** the identified FOXO1 DNA binding site in the PIGF promoter and **(f)** region containing the TATAA box proximal to the PIGF transcription start site, relative to negative control DNA region (NegC) using ChIP assays. Results are the mean (± SEM) of ≥ 3 independent experiments and analysed using **(b–d)** Student's t-test and **(e,f)** one-way ANOVA, Tukey's post hoc test; *P < 0.05, **P < 0.01.

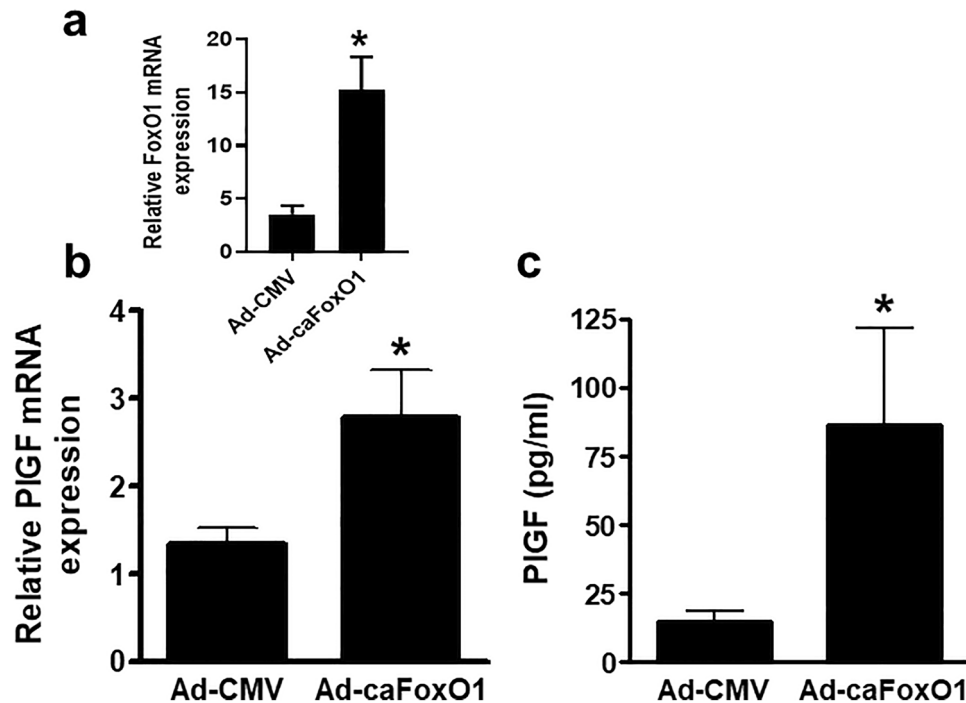


Figure 5. FOXO1 promotes PIGF expression in vivo. FVB/N mice were injected via the tail vein with 2×10^{10} infective units of Ad-caFoxO1, or Ad-CMV control virus (8 mice per group). After 48 h the mice were euthanised and blood and liver tissue collected. (a) PIGF and FoxO1 mRNA expression quantified in the liver by qPCR and (b) circulating PIGF was measured in plasma by ELISA. Results are the mean \pm SEM of 8 mice per group; Student's t test, * $P < 0.05$.

of Akt in the vasculature³⁰ and its loss leads to severe atherosclerosis in ApoE^{-/-} mice⁶. It is interesting to note that PCR array data from the same study, indicate that PIGF is up-regulated in mice fed a high fat diet following deletion of Akt1 on an ApoE^{-/-} background⁶.

In contrast to our findings, a study using bovine retinal endothelial cells found that hyperglycaemia induced PIGF expression indirectly via an autocrine VEGF-dependent mechanism, based on the use of a VEGF receptor-2 inhibitor²⁷. In our hands, VEGF knock-down in HUVEC did not reduce PIGF expression, as would be expected if PIGF production was secondary to the induction of endogenous VEGF. On the contrary, loss of endogenous VEGF expression was found to promote PIGF expression and secretion significantly (Fig. S2). Furthermore, the observed time-frame of PIGF mRNA induction in response to hyperglycaemia in this study was not consistent with PIGF release dependent on the up-regulation of VEGF expression. However, our findings are in line with more recent studies which show that endogenous VEGF suppresses FOXO1 activity in endothelial cells in an autocrine manner^{31,32}. Finally, PIGF up-regulation following stimulation of endothelial cells with recombinant VEGF was reported to be via PKC signalling and independent of PIGF gene transcription³³.

We have previously examined the level of PIGF production in many human primary and transformed cell types, and apart from trophoblast-derived cell lines (HTR-8/pSVneo, JAR) and thyroid cells the most significant source appears to be both microvascular and large vessel endothelial cells. Indeed, PIGF was reported to be the most abundant VEGF family member expressed in human dermal microvascular endothelium, detected at 100-fold greater levels than the least abundant, VEGF-A³⁴. In murine fibroblasts and a human choriocarcinoma cell line, PIGF was reported to be induced by hypoxia through the metal-responsive transcription factor, MTF-1^{35,36}. PIGF is switched on selectively in the embryonic kidney by the epithelium-specific BF-2 (FOXD2) transcription factor³⁷, and the forkhead-related transcription factor, glial cell missing-1 (GCM-1), regulates PIGF expression in trophoblast cell lines^{38,39}. In addition, heme-bound iron has been shown to up-regulate PIGF in erythroid cells via the erythroid krüppel-like factor (EKLF/KLF-1)⁴⁰. However, there is a paucity of information on the control of PIGF expression in endothelial cells. Using siRNA-mediated knock-down or over-expression of constitutively active FOXO1, we demonstrate that PIGF expression is regulated directly by FOXO1 activity in endothelial cells. Furthermore, the increase in PIGF release in response to hyperglycaemia was lost in HUVEC following knock-down of FOXO1, and no IGF-1-mediated suppression of PIGF production was observed in the presence of constitutively active FOXO1. Moreover, FOXO-1 binding was detected at a FOXO-1 consensus DNA binding sequence identified in the PIGF promoter, and enriched ~fourfold following exposure of the cells to hyperglycaemia. In contrast to our findings, the acute release of PIGF, assessed using protein arrays, from HAEC within one hour was reported to be associated with a loss of FOXO1 activity through C5b-9 complement complex activation of Akt⁴¹. Conversely, in the same study FOXO1 knock-down actually prevented acute PIGF protein secretion, suggesting a differential regulatory role of FOXO1 in the transcription and secretion/release of PIGF⁴¹. FOXO1 is the predominant FOXO family member expressed in the vasculature, liver and pancreas and is associated

with glucose metabolism and the regulation of insulin sensitive genes^{5,42,43}. FOXO1 haploinsufficiency in mice protects against obesity-related insulin resistance⁴⁴ and diet-induced diabetes⁴⁵, and in rat models of diabetes, FOXO1 knock-down in the vitreous reduces retinal endothelial apoptosis in hyperglycaemia²⁶. Furthermore, up-regulation of FOXO1 activity in endothelial cells exposed to hyperglycaemia has been identified as a key response promoting oxidative stress through the up-regulation of inducible NO synthase⁴⁶. In addition to decreasing the level of FOXO1 phosphorylation this study showed that hyperglycaemia also reduces the level of FOXO1 acetylation which is associated increased transcriptional activity⁴⁶. The significance of endothelial FOXO1 activity in the promotion of atherosclerosis associated with insulin resistance has also been highlighted^{5,47}.

FOXO transcription factors are important modulators of vessel formation, maturation, maintenance and homeostasis^{5,42,43,47–49}. Indeed, FOXO1-deficient mice die in utero from embryonic day 9.5, exhibiting vascular defects in the aortic and branchial arches and failure to form blood vessels in the yolk sac^{48,49}. FOXO1 was reported to be essential for in vitro vascular formation from embryonic stem cells⁵⁰, and over-expression or knock-down of either FOXO1 or FOXO3A in HUVEC leads to increased endothelial cell migration and capillary-like tube formation on growth factor-reduced Matrigel⁵⁰. Recently the importance of FOXO1 in regulating endothelial cell quiescence has also been highlighted^{43,49}. Several genes involved in angiogenesis, vascular remodelling and maintenance/homeostasis, including angiopoietin-2 (Ang-2) are regulated by FOXO1, but not FOXO3A which is associated with apoptosis^{42,51}. Similarly, we find that FOXO3A does not regulate PIGF mRNA expression or protein release. Interestingly, microarray data from these studies and others indicate that FOXO1 also regulates VEGFR-1 expression in endothelial cells^{42,51,52}. In pathologies, both PIGF and VEGFR-1 are frequently up-regulated in the endothelium¹¹, and therefore the increased FOXO1 activity which is often observed in these conditions may provide a common mechanism by which both receptor and ligand are up-regulated to promote PIGF – VEGFR-1 signalling.

Insulin resistance, hyperglycaemia and diabetes are frequently associated with obesity, and are characteristics of the metabolic syndrome. In a cross-sectional survey conducted in three isolated populations in Southern Italy of 1000 men and women, circulating PIGF levels were significantly higher in subjects with the metabolic syndrome and correlated with the number of metabolic criteria in these patients²⁰. In addition, circulating PIGF is associated with obesity in children and correlated with troponin levels²². PIGF mRNA is expressed in white adipose tissue³³, and PIGF^(-/-) knock-out mice have a lower body weight and fat content than wild-type mice and a lower fat content when maintained on a high-fat diet⁵⁴. Whether this is due to endothelial or adipocyte PIGF production was not fully established, but it suggests that in addition to increased risk of type II diabetes²¹ and CVD^{19,23–25}, up-regulation of PIGF in insulin resistance/hyperglycaemia and the metabolic syndrome may also accelerate the development of obesity.

PIGF is a key inflammatory mediator that activates monocytes, promoting migration and inflammatory cytokine and tissue factor expression^{11,55,56}. It is suggested that a major component of PIGF activity in pathologies may be through the induction of inflammatory infiltrates, rather than direct activation of the endothelium¹¹. However, both PIGF and VEGFR-1 are frequently up-regulated in the endothelium in pathology¹¹, and whilst it is established that the majority of endothelial VEGF responses are mediated via VEGFR-2, evidence from our laboratory and others has demonstrated that VEGFR-1 can act as a direct signalling receptor in endothelial cells^{57–59}, and PIGF-mediated VEGFR-1 activation leads to distinct endothelial gene expression to that of VEGF¹². The discovery that the PI3K/Akt/FOXO1 signalling axis regulates PIGF expression in endothelium is likely to be important for its pathological activity in diabetic retinopathy, atherosclerosis, and have wider implications in obesity and the metabolic syndrome, which are all characterised by endothelial dysfunction. Thus, it may represent a unifying pathway by which PIGF signalling may be up-regulated in these conditions and provide the basis for new approaches to limit its activity.

Methods

Cell culture. Human umbilical vein endothelial cells (HUVEC) were either isolated, characterised and cultured essentially as described previously⁵⁷ from umbilical cords obtained following informed consent under ethical approval [15-014-014] of the Human Biomaterials Resource Centre, University of Birmingham, UK. Pooled HUVEC from multiple donors were also purchased from PromoCell and human aortic endothelial cells (HAEC) from Lonza and maintained in their endothelial cell growth media and according to the supplier's recommendations. Endothelial cells were routinely plated on 0.1% gelatin-coated tissue culture flasks/plates and experiments were performed using cells at the third or fourth passage. For experiments to examine PIGF secretion endothelial cells (1×10^5 cells/well) were plated in 24-well plates with 0.5 ml of growth medium to produced confluent monolayers and incubated for 24 h. The medium was then replaced with 0.5 ml MCDB131 (Invitrogen) containing 10% FCS and cells incubated for a further 24 h. The medium was then harvested and centrifuged at $1000 \times g$ for 5 min and supernatant frozen at -80°C prior to ELISA analysis. To examine the effect of hyperglycaemia cells were incubated in MCDB131 medium (Gibco) containing 10% FCS and a final concentration of 5 mM D-glucose (NG = normal glycaemia), 30 mM D-glucose (HG = hyperglycaemia), or 25 mM L-glucose + 5 mM D-glucose (OS = osmolarity control). Recombinant IGF-1 (Peprotech EC, (London, UK) and the PI3 kinase (PI3K) inhibitor, LY294002 (Calbiochem, Nottingham, UK) were added as indicated. MTT assays were performed to ensure that there was no effect of the treatments on cell viability.

Adenoviruses. The recombinant replication-deficient adenoviruses encoding constitutively active ADA mutant of FoxO1 (Ad-caFoxO1) was a kind gift of Professor D Acilli, Columbia University, NY, USA and dominant-negative Akt (Ad-dn-Akt), myristoylated Akt (Ad-myr-Akt)⁶⁰, PTEN, and PTENc/s were a kind gift of Dr Christopher Kontos, Duke University, USA. All adenoviruses were amplified in HEK-293 cells and isolated from the cells following 4 snap freeze/thaw cycles by CsCl gradient centrifugation and dialysed into 10 mM Tris HCl

(pH 8.0), 2 mM MgCl₂, 4% sucrose and stored at – 80 °C. Purified adenoviruses were titred using the Adeno-X™ Rapid Titer Kit (Takara) and the optimal multiplicity of infection determined by Western blotting as we described previously^{57,61}. HUVEC were infected with 50–100 infective units (IFU)/cell and incubated for 18 h in MCDDB131 medium containing 5% FCS prior to the experiment.

Experimental animals. All procedures involving animals were performed in accordance with protocols and regulations approved by the University of Birmingham Animal Welfare Ethics Review Body, under a licence issued in accordance with the Animals (Scientific Procedures) Act 1986, adopting the principles of the 3Rs (Replacement, Reduction and Refinement), and carried out in compliance with the ARRIVE guidelines for animal study (<http://www.nc3rs.org.uk/page.asp?id=1357>).

FVB/N mice at 10-to-12 weeks of age were housed in a temperature, humidity, and light-controlled room with a 12-h light/dark cycle and allowed free access to water and food. The mice were divided into two age- and sex-matched groups (8 mice per group), housed in different cages, and injected via the tail vein with recombinant adenovirus (2 × 10¹⁰ IFU) encoding caFoxO1 or Ad-CMV empty control virus. After 48 h the mice were euthanized and blood was collected by cardiac puncture to obtain plasma, and the livers snap-frozen for the preparation of mRNA, by an individual blinded to the original treatment.

siRNA-mediated gene knock-down. siRNAs targeted to human FOXO1, FOXO3A, AKT1 and VEGF were introduced into HUVEC using the Amaxa Nucleofector HUVEC II kit according (Amaxa) as we described previously⁶¹, or Lipofectamine RNAiMax transfection reagent (Thermo Fisher) following the manufacturer's recommended protocol. Following transfection the cells were incubated for 18 h overnight prior to further treatment. The siRNA sequences were selected based on previously reported efficacy as follows: FOXO1, 5'-AAG AGCGUGCCCUACUUAAG-3'^{42,49}; FOXO3A, 5'-AAGAGCUCUUGGU GGAUCAUC-3'^{42,49}; VEGF, 5'-AAG GAGUACCCUGAUGAGAUC-3'⁶²; Akt1, 5'-AAGGAGGGUUGGUGCACAAC-3'⁶³; A universal control siRNA from Dharmacon was used as a negative control.

ELISA. Human and mouse PlGF levels were measured in cell supernatants using DuoSet IC ELISA kits according to the manufacturer's instructions (R&D Systems). Results were expressed either as the mean concentration of PlGF in pg/ml of conditioned medium, or the "mean % release" calculated as a percentage of each sample relative to the untreated control for each experiment.

RNA isolation and real-time qPCR. The Total RNA plus kit (Norgen Biotek Corp, Canada) was used to isolate RNA from cell and tissues according to the manufacturer's instructions. Following stimulation, the medium was aspirated and cell monolayers were washed twice with ice-cold PBS and lysed directly in buffer RL. Mouse tissues were disaggregated in ice-cold buffer RL using a Precellys 24 tissue homogenizer set at 2 cycles a 6500 rpm for 20 s with a 30 s interval at 4 °C. Total RNA was extracted and subjected to DNase-1 digestion and ~ 1 µg was reverse transcribed with oligo-dT₁₈ primers using the First Strand Synthesis Kit (Promega) for 1 h at 48 °C. Real-time qPCR was performed using a Rotagene RG-3000 (Qiagen) under the following amplification conditions: 95 °C for 10 min, followed by 45 cycles of 95 °C, 10 s; 58 °C, 10 s; 72 °C, 20 s. cDNA samples were amplified in triplicate using SensiMix SYBR green (Bioline) and primers specific for human and mouse PlGF, FOXO1, FOXO3A, VEGF and β-actin (Table S1). PCR products were analysed by 2% agarose gel electrophoresis, sequenced, and post-run melt curve analysis performed to ensure specificity of reactions. The mean threshold cycle (Ct) for each sample was normalised to β-actin levels and fold changes to the experimental control calculated using the $\Delta\Delta C_t$ method.

Western blotting. Cell monolayers were washed twice with ice-cold PBS and lysed directly in ice-cold RIPA buffer (Pierce) containing a phosphatase and complete protease inhibitors (Roche) and a total of 30 µg protein of each sample run on 10% SDS-PAGE gels, and blotted onto nitrocellulose or PVDF membranes as described⁵⁷. Membranes were blocked in TBS-T (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) containing 5% (w/v) fat-free milk for 1–2 h and incubated with primary antibodies at the concentration indicated (Table S2) in TBS-T containing 5% BSA overnight at 4 °C. After washing in TBS-T (4 × 10 min), the blots were incubated for 1 h with a 1:10,000 dilution of the appropriate horseradish peroxidase-conjugated antibody (Cell Signalling Technologies) in TBS-T containing 5% (w/v) fat-free milk for 1 h at room temperature and proteins detected by enhanced chemiluminescence. Following the detection of phosphorylated Akt, or FOXO1 membranes were stripped in NaOH (200 mM) for 20 min at room temperature and re-probed for total Akt or FOXO1 protein respectively and β-actin, or α/β tubulin used as a loading control.

CHIP assays. Endothelial cells were grown to ~ 80% confluence 15 cm culture dishes and treated under the conditions indicated under Cell Culture above. The cells (~ 1 × 10⁷/sample) were fixed/chromatin cross-linked for 10 min using methanol-free formaldehyde (Pierce) added directly to the culture medium to a final concentration of 1%⁶⁴. The formaldehyde was then quenched by adding 2 M glycine to a final concentration of 125 mM and cells washed twice with ice-cold PBS^{64,65}. The cells were then scrapped into ice-cold PBS, pelleted and lysed in 50 mM HEPES (pH 8.0) containing, 150 mM NaCl, 10 mM EDTA, 10% glycerol, 0.25% Triton X-100 and complete protease inhibitor cocktail (Roche) and incubated for 10 min at 4 °C with end-over-end rotation^{64,65}. Nuclei were collected by centrifugation and resuspended in ice-cold wash buffer (10 mM HEPES, 10 mM EDTA, 0.5 mM EGTA, 0.25% Triton X-100, (pH 8), complete protease inhibitors on a rotary mixer. The nuclei were sonicated on ice using Bioruptor (Diagenode, USA) set on "high" (240 W) with 20–25 cycles of 30

secs ON; 30 secs OFF to give fragmented DNA of ~100–300 bp. The sonicated material was then centrifuged for 10 min at 16,000×g at 4 °C and the supernatants harvested. Chromatin was diluted with CHIP buffer (25 mM Tris–HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.25% SDS, 0.1 mM PMSF and complete protease inhibitors⁶⁵) and 50 µL was kept as the “input” and the rest immunoprecipitated using 10 µg of rabbit anti-FOXO1 (H-128, Santa Cruz Biotechnology, USA), rabbit anti-RNA Pol II (ab26721, Abcam, UK), or isotype-matched IgG as positive and negative controls respectively. The antibody was added with BSA (0.5% final concentration) to 10 µL of protein A/G magnetic beads (Pierce), following washing and resuspension in 100 mM phosphate buffer, and incubated for 2 h at 4 °C on a rotary mixer. The chromatin cross-links were reversed by adding 1 µL of RNase A (10 mg/ml) with 30 min incubation. Chromatin was subjected to qPCR in triplicate with primers flanking the putative FOXO1 DNA binding sequence identified in the *PIGF* promoter (5'-AGAGCA CCAAGCACACCTGTTT-3 and 5'-CAGTGCCCAACTCAACAC-3') and proximal to the *PIGF* transcriptional start site (5'-CCATTCGACATATGCAGGCA-3' and 5'-CATGATCTAACCGCCTCTGC-3'). A negative control region (NegC; ActiveMotif, UK) was used to calculate the relative enrichment. Input chromatin was used to generate an amplification curve for each primer set to determine the amount of DNA immunoprecipitated with anti-FOXO1, Pol II and isotype-matched IgG control antibodies. Binding data are presented as the mean fold-enrichment at the putative FOXO1 consensus site, or adjacent to the *PIGF* transcriptional start site, relative to the negative control region (NegC; ActiveMotif) based on the percentage of input obtained.

Statistical analysis. All data are expressed as the mean ± SD or SEM as indicated. Statistical analysis was performed with GraphPad PRISM using either an unpaired, two-tailed Student's *t*-test, or non-parametric one-way ANOVA with Tukey's multiple comparison post hoc test. A *P* value of <0.05 was considered statistically significant (**P* < 0.05; ***P* < 0.01).

Received: 6 July 2020; Accepted: 13 July 2021
Published online: 11 August 2021

References

1. Grundy, S. M. *et al.* Prevention Conference VI: Diabetes and Cardiovascular Disease: Executive summary: Conference proceeding for healthcare professionals from a special writing group of the American Heart Association. *Circulation* **105**, 2231–2239 (2002).
2. Ginsberg, H. N. Insulin resistance and cardiovascular disease. *J. Clin. Invest.* **106**, 453–458 (2000).
3. Holman, R. R., Paul, S. K., Bethel, M. A., Matthews, D. R. & Neil, H. A. 10-year follow-up of intensive glucose control in type 2 diabetes. *N. Engl. J. Med.* **359**, 1577–1589 (2008).
4. Du, X. L. *et al.* Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 12222–12226 (2000).
5. Kamagate, A. *et al.* FoxO1 mediates insulin-dependent regulation of hepatic VLDL production in mice. *J. Clin. Invest.* **118**, 2347–2364 (2008).
6. Fernandez-Hernando, C. *et al.* Loss of Akt1 leads to severe atherosclerosis and occlusive coronary artery disease. *Cell Metab.* **6**, 446–457 (2007).
7. Du, X. L. *et al.* Hyperglycemia inhibits endothelial nitric oxide synthase activity by posttranslational modification at the Akt site. *J. Clin. Invest.* **108**, 1341–1348 (2001).
8. Ito, H. *et al.* Endothelial progenitor cells as putative targets for angiostatin. *Cancer Res.* **59**, 5875–5877 (1999).
9. Du, K., Herzig, S., Kulkarni, R. N. & Montminy, M. TRB3: A tribbles homolog that inhibits Akt/PKB activation by insulin in liver. *Science* **300**, 1574–1577 (2003).
10. Butler, M. *et al.* Specific inhibition of PTEN expression reverses hyperglycemia in diabetic mice. *Diabetes* **51**, 1028–1034 (2002).
11. Carmeliet, P. *et al.* Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. *Nat. Med.* **7**, 575–583 (2001).
12. Autiero, M. *et al.* Role of PIGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. *Nat. Med.* **9**, 936–943 (2003).
13. Khaliq, A. *et al.* Increased expression of placenta growth factor in proliferative diabetic retinopathy. *Lab. Invest.* **78**, 109–116 (1998).
14. Mitamura, Y. *et al.* Vitreous levels of placenta growth factor and vascular endothelial growth factor in patients with proliferative diabetic retinopathy. *Diabetes Care* **25**, 2352 (2002).
15. Miyamoto, N. *et al.* Placental growth factor-1 and epithelial haemato-retinal barrier breakdown: Potential implication in the pathogenesis of diabetic retinopathy. *Diabetologia* **50**, 461–470 (2007).
16. Cao, R. *et al.* VEGFR1-mediated pericyte ablation links VEGF and PIGF to cancer-associated retinopathy. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 56–861 (2009).
17. Ideno, J. *et al.* Prevention of diabetic retinopathy by intraocular soluble flt-1 gene transfer in a spontaneously diabetic rat model. *Int. J. Mol. Med.* **19**, 75–79 (2007).
18. Van Bergen, T. *et al.* Neutralization of placental growth factor as a novel treatment option in diabetic retinopathy. *Exp. Eye Res.* **165**, 136–150. <https://doi.org/10.1016/j.exer.2017.09.012> (2017).
19. Lenderink, T. *et al.* Elevated placental growth factor levels are associated with adverse outcomes at four-year follow-up in patients with acute coronary syndromes. *J. Am. Coll. Cardiol.* **47**, 307–311 (2006).
20. Siervo, M. *et al.* Angiogenesis and biomarkers of cardiovascular risk in adults with metabolic syndrome. *J. Intern. Med.* **268**, 338–347 (2010).
21. Duran, E. K. *et al.* Plasma placental growth factor concentrations are elevated well in advance of type 2 diabetes mellitus onset: Prospective data from the WHS. *J. Am. Heart Assoc.* **8**, e012790. <https://doi.org/10.1161/JAHA.119.012790> (2019).
22. Pervanidou, P. *et al.* Increased placental growth factor (PIGF) concentrations in children and adolescents with obesity and the metabolic syndrome. *Hormones (Athens)* **13**, 369–374. <https://doi.org/10.14310/horm.2002.1491> (2014).
23. Pilarczyk, K. *et al.* Placenta growth factor expression in human atherosclerotic carotid plaques is related to plaque destabilization. *Atherosclerosis* **196**, 333–340 (2008).
24. Khurana, R. *et al.* Placental growth factor promotes atherosclerotic intimal thickening and macrophage accumulation. *Circulation* **111**, 2828–2836 (2005).
25. Roncal, C. *et al.* Short-term delivery of anti-PIGF antibody delays progression of atherosclerotic plaques to vulnerable lesions. *Cardiovasc. Res.* **86**, 29–36 (2009).

26. Behl, Y., Krothapalli, P., Desta, T., Roy, S. & Graves, D. T. FOXO1 plays an important role in enhanced microvascular cell apoptosis and microvascular cell loss in type 1 and type 2 diabetic rats. *Diabetes* **58**, 917–925 (2009).
27. Zhao, B., Cai, J. & Boulton, M. Expression of placenta growth factor is regulated by both VEGF and hyperglycaemia via VEGFR-2. *Microvasc. Res.* **68**, 239–246 (2004).
28. Brunet, A. *et al.* Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* **96**, 857–868 (1999).
29. Shao, J., Yamashita, H., Qiao, L. & Friedman, J. E. Decreased Akt kinase activity and insulin resistance in C57BL/KsJ-Lepr^{db/db} mice. *J. Endocrinol.* **167**, 107–115 (2000).
30. Chen, J. *et al.* Akt1 regulates pathological angiogenesis, vascular maturation and permeability in vivo. *Nat. Med.* **11**, 1188–1196 (2005).
31. Domigan, C. K. *et al.* Autocrine VEGF maintains endothelial survival through regulation of metabolism and autophagy. *J. Cell Sci.* **128**, 2236–2248. <https://doi.org/10.1242/jcs.163774> (2015).
32. Lee, S. *et al.* Autocrine VEGF signaling is required for vascular homeostasis. *Cell* **130**, 691–703 (2007).
33. Yao, Y. G., Yang, H. S., Cao, Z., Danielsson, J. & Duh, E. J. Upregulation of placental growth factor by vascular endothelial growth factor via a post-transcriptional mechanism. *FEBS Lett.* **579**, 1227–1234 (2005).
34. Yonekura, H. *et al.* Placenta growth factor and vascular endothelial growth factor B and C expression in microvascular endothelial cells and pericytes. Implication in autocrine and paracrine regulation of angiogenesis. *J. Biol. Chem.* **274**, 35172–35178 (1999).
35. Green, C. J. *et al.* Placenta growth factor gene expression is induced by hypoxia in fibroblasts: A central role for metal transcription factor-1. *Cancer Res.* **61**, 2696–2703 (2001).
36. Nishimoto, F. *et al.* Metal transcription factor-1 is involved in hypoxia-dependent regulation of placenta growth factor in trophoblast-derived cells. *Endocrinology* **150**, 1801–1808 (2009).
37. Zhang, H. *et al.* Transcriptional activation of placental growth factor by the forkhead/winged helix transcription factor FoxD1. *Curr. Biol.* **13**, 1625–1629 (2003).
38. Chang, M. *et al.* Glial cell missing 1 regulates placental growth factor (PGF) gene transcription in human trophoblast. *Biol. Reprod.* **78**, 841–851 (2008).
39. Chiu, Y. H. *et al.* New insights into the regulation of placental growth factor gene expression by the transcription factors GCM1 and DLX3 in human placenta. *J. Biol. Chem.* **293**, 9801–9811. <https://doi.org/10.1074/jbc.RA117.001384> (2018).
40. Wang, X. *et al.* Heme-bound iron activates placenta growth factor in erythroid cells via erythroid Kruppel-like factor. *Blood* **124**, 946–954. <https://doi.org/10.1182/blood-2013-11-539718> (2014).
41. Fosbrink, M., Niculescu, F., Rus, V., Shin, M. L. & Rus, H. C5b-9-induced endothelial cell proliferation and migration are dependent on Akt inactivation of forkhead transcription factor FOXO1. *J. Biol. Chem.* **281**, 19009–19018 (2006).
42. Potente, M. *et al.* Involvement of Foxo transcription factors in angiogenesis and postnatal neovascularization. *J. Clin. Invest.* **115**, 2382–2392 (2005).
43. Wilhelm, K. *et al.* FOXO1 couples metabolic activity and growth state in the vascular endothelium. *Nature* **529**, 216–220. <https://doi.org/10.1038/nature16498> (2016).
44. Kim, J. J. *et al.* FoxO1 haploinsufficiency protects against high-fat diet-induced insulin resistance with enhanced peroxisome proliferator-activated receptor gamma activation in adipose tissue. *Diabetes* **58**, 1275–1282 (2009).
45. Nakae, J. *et al.* The forkhead transcription factor Foxo1 regulates adipocyte differentiation. *Dev. Cell* **4**, 119–129 (2003).
46. Tanaka, J. *et al.* Foxo1 links hyperglycemia to LDL oxidation and endothelial nitric oxide synthase dysfunction in vascular endothelial cells. *Diabetes* **58**, 2344–2354 (2009).
47. Tsuchiya, K. *et al.* Homozygosity for an allele encoding deacetylated FoxO1 protects macrophages from cholesterol-induced inflammation without increasing apoptosis. *Arterioscler. Thromb. Vasc. Biol.* **31**, 2920–2928 (2011).
48. Furuyama, T. *et al.* Abnormal angiogenesis in Foxo1 (Fkhr)-deficient mice. *J. Biol. Chem.* **279**, 34741–34749 (2004).
49. Dharaneeswaran, H. *et al.* FOXO1-mediated activation of Akt plays a critical role in vascular homeostasis. *Circ. Res.* **115**, 238–251. <https://doi.org/10.1161/CIRCRESAHA.115.303227> (2014).
50. Park, S. H., Sakamoto, H., Tsuji-Tamura, K., Furuyama, T. & Ogawa, M. Foxo1 is essential for in vitro vascular formation from embryonic stem cells. *Biochem. Biophys. Res. Commun.* **390**, 861–866 (2009).
51. Daly, C. *et al.* Angiopoietin-1 modulates endothelial cell function and gene expression via the transcription factor FKHR (FOXO1). *Genes Dev.* **18**, 1060–1071 (2004).
52. Taddei, A. *et al.* Endothelial adherens junctions control tight junctions by VE-cadherin-mediated upregulation of claudin-5. *Nat. Cell Biol.* **10**, 923–934 (2008).
53. Hagberg, C. E. *et al.* Vascular endothelial growth factor B controls endothelial fatty acid uptake. *Nature* **464**, 917–921 (2010).
54. Lijnen, H. R. *et al.* Impaired adipose tissue development in mice with inactivation of placental growth factor function. *Diabetes* **55**, 2698–2704 (2006).
55. Clauss, M. *et al.* The vascular endothelial growth factor receptor Flt-1 mediates biological activities. Implications for a functional role of placenta growth factor in monocyte activation and chemotaxis. *J. Biol. Chem.* **271**, 17629–17634 (1996).
56. Selvaraj, S. K. *et al.* Mechanism of monocyte activation and expression of proinflammatory cytochemokines by placenta growth factor. *Blood* **102**, 1515–1524 (2003).
57. Ahmad, S. *et al.* Direct evidence for endothelial vascular endothelial growth factor receptor-1 function in nitric oxide-mediated angiogenesis. *Circ. Res.* **99**, 715–722 (2006).
58. Bussolati, B. *et al.* Vascular endothelial growth factor receptor-1 modulates vascular endothelial growth factor-mediated angiogenesis via nitric oxide. *Am. J. Pathol.* **159**, 993–1008 (2001).
59. Zeng, H., Zhao, D. & Mukhopadhyay, D. Flt-1-mediated down-regulation of endothelial cell proliferation through pertussis toxin-sensitive G proteins, beta gamma subunits, small GTPase CDC42, and partly by Rac-1. *J. Biol. Chem.* **277**, 4003–4009 (2002).
60. Fulton, D. *et al.* Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* **399**, 597–601 (1999).
61. Cudmore, M. J. *et al.* Loss of Akt activity increases circulating soluble endoglin release in preeclampsia: Identification of interdependency between Akt-1 and heme oxygenase-1. *Eur. Heart J.* **33**, 1150–1158 (2012).
62. Takei, Y., Kadomatsu, K., Yuzawa, Y., Matsuo, S. & Muramatsu, T. A small interfering RNA targeting vascular endothelial growth factor as cancer therapeutics. *Cancer Res.* **64**, 3365–3370 (2004).
63. Jiang, Z. *et al.* Phosphatase and tensin homologue deficiency in glioblastoma confers resistance to radiation and temozolomide that is reversed by the protease inhibitor nelfinavir. *Cancer Res.* **67**, 4467–4473 (2007).
64. Sissaoui, S. *et al.* Genomic characterization of endothelial enhancers reveals a multifunctional role for NR2F2 in regulation of arteriovenous gene expression. *Circ. Res.* **126**, 875–888. <https://doi.org/10.1161/CIRCRESAHA.119.316075> (2020).
65. Kershaw, R. M., Siddiqui, Y. H., Roberts, D., Jayaraman, P. S. & Gaston, K. PRH/HHex inhibits the migration of breast and prostate epithelial cells through direct transcriptional regulation of Endoglin. *Oncogene* **33**, 5592–5600. <https://doi.org/10.1038/ncr.2013.496> (2014).

Acknowledgements

This work was supported by grants from the Medical Research Council (G0601295 and G0700288) and Birmingham Women's Hospital Trust, Birmingham UK. S.S. was supported by a Medical Research Council DTP studentship. The authors wish to thank Dr Margaret Eggo for her considerable help and advice during the study.

Author contributions

Study conception and design S.S., A.A., and P.W.H.; performed experiments and data analysis; S.S., L.T., S.E. and P.W.H.; manuscript drafted by P.W.H.; critical manuscript revisions P.W.H. and S.E. All Authors reviewed the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-021-95511-8>.

Correspondence and requests for materials should be addressed to P.W.H.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2021