Molecular Regulation of Placental Growth Factor (PlGF) Expression in Endothelial Cells

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This PhD thesis written in 2011 is dedicated:

To My beloved parents, Halima and Tahar

And to all my brothers, sisters and their families.

Abstract

Placental growth factor (PlGF) is a pro-angiogenic and inflammatory mediator that promotes many pathological conditions including, diabetes, atherosclerosis and cancer. In mouse models, the loss of PlGF or inhibition of vascular endothelial growth factor receptor-1 (VEGFR-1) activity suppresses these disorders. Hyperglycaemia plays a fundamental role in the pathogenesis of type-2 diabetes and associated conditions, resulting in a loss of PI3 kinase (PI3K) signalling and dysfunction in endothelial cells. Using pharmacological inhibitors, siRNA, and adenoviral constructs to modulate the PI3K/Akt signalling activity, I found that the induction of PlGF expression in human umbilical vein endothelial cells (HUVEC) by hyperglycaemia is PI3K/Akt-dependent. Using similar approaches, the FOXO1 transcription factor was identified as the downstream target of Akt involved in the regulation of both PlGF and VEGFR-1 expression. FOXO1 was found to interact directly with the VEGFR-1 gene promoter in vitro, and over-expression of constitutively-active FOXO1 promotes PlGF expression *in vivo*. Although VEGF activates PI3K/Akt, it stimulates robust PlGF release in endothelial cells. Here I show that this effect is both VEGFR-2 and PKC-dependent, but independent of PI3K/Akt. The PI3K/Akt/FOXO1 axis is an important regulator of vascular homeostasis and stress responses and the identification of its involvement in PlGF expression may provide new therapeutic targets for disorders characterised by endothelial dysfunction.

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List of publications

Published Articles

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Ad	Non-replicative, recombinant adenovirus.
AGE	Advanced glycation end products
ApoCIII	Apoliproprotein CIII
ApoE$^{-/-}$	apolipoprotein-E-deficient
aPKC	Abnormal protein kinase C
BAEC	Bovine aortic endothelial cells
Bp	Base pairs
bFGF	Basic fibroblasts growth factor
BSA	Bovine serum albumin
BWH	Birmingham women's hospital
CREB	cAMP response element-binding
CBP	CREB-binding protein
ChIP	Chromatin immunoprecipitation
cpw	Cells per well
CMV	Cytomegalovirus
CVD	Cardiovascular disease
DAB	3,3'-diaminobenzidine in chromogen solution
DAF-16	Abnormal daur formation factor-16
DAG	Diacylglycerol
DBD	DNA-Binding-Domain
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
Dn-	Dominant-negative
DTT	Dithiothreitol
EC	Endothelial cells
ECL	Enhanced chemiluminescence

ECM	Extracellular matrix
EGF	Epidermal growth factor
Egr-1	Early growth response factor-1
ELISA	Enzyme-Linked Immunosorbent Assay
eNOS	Endothelial nitric oxide synthase
EPC	Endothelial progenitor cells
ETS	ETS-domain transcription factors
Erg	ETS-related gene
ERK	Extracellular signal regulated kinase a.k.a MAPK
ET-1	Endothelin-1
FAT	Factor acetyl transferase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FKH	Forkhead domain
FOXO	Forkhead boxO
FRE	FOXO-recognition element
g	Gravity
G6Pase	Glucose-6-phosphatase
GPCRs	G protein-coupled receptors
G/P/S	L-Glutamine, 10U/ml penicillin and 0.1 µg/ml streptomycin
GLUT-4	Glucose transporter type 4
HAEC	Human aortic endothelial cells
HBSS	Hank’s balanced saline solution
HEK 293	Human embryonic kidney 293 cells
ICAM-1	Inter-Cellular Adhesion Molecule 1
IGFPB	Iinsulin-like-growth factor binding proteins
HIF-1	Hypoxia Inducible Factor-1

H_2O_2	Hydrogen peroxide
HRG	Histidine-rich glycoprotein
HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cells
HG	Hyperglycaemia
ifu	Infectious unit
IG	Immunoglobulin
IGF-1	Insulin-like growth factor-1
IGF-IR	Insulin-like growth factor-1 receptor
IP	Immunoprecipitation
IR	Insulin receptor
IRS	Insulin/IGF-I receptor substrates
IL-2	intreleukin-2
IL-6	interleukin-6
JNK	c-Jun N-terminal kinases
kDa	Kilo Dalton
LPL	Lipoprotein lipase
M199	Medium 199
MAFbx	Muscle atrophy F box
MBS	metabolic syndrome
MEK	Map kinase kinase
MOI	Multiplicity of infection
MnSOD	Manganese superoxide dismutase
mTORC2	mTOR complex 2
MuRF1	Atrogin-1 and muscle RING finger 1
Myr-	Myristoylated
NES	Nuclear export sequence

NLS	Nuclear localisation signal
NF-KB	Nuclear Factor-k B
NG	Normoglycaemia
NO	Nitric oxide
ob/ob	Obese mouse
OD	Optical density
OS	Osmolarity control
NP-1 & NP-2	Neuropilin-1 & -2.
PAI-I	Plasminogen activator inhibitor-I
PBS	Phosphate Buffered Saline
PDGF	Platelet-derived growth factor
PDK1	Phosphoinositide-dependent protein kinase 1
PEPCK	Phosphoenolpyruvate carboxykinase
PFDX1	Pancreatic and duodenal homeobox 1
PH	Pleckstrin homology
PI3K	Phosphoinositide 3-kinase
PI(3)P	Phosphatidylinositol 3-phosphate
PIP2	Phosphatidylinositol (3,4)-bisphosphate ($PI(3,4)P_2$)
PIP3	phosphatidylinositol (3,4,5)-trisphosphate ($PI(3,4,5)P_3$
PlGF	Placental growth factor
PKB	Protein kinase B
PKC	protein kinase C
PLCγ	phospholipase Cγ
PPARγ	Peroxisome proliferator-activated receptor gamma
PTEN	Phosphatase and tensin homolog on chromosome 10
PTPases	Phosphatases
qPCR	Quantitative polymerase chain reaction

RA	Rheumatoid arthritis
MAPK	Mitogen-activated protein kinase
RIPA	Radio immunoprecipitation assay
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium cells
RT	Room temperature
RTKs	Receptor tyrosine kinases
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
siRNA	Short interfeing RNA
SGK	serum-and glucocorticoid-induced protein kinase
SOCS1/3	Suppressor of cytokine signalling-1/3
SOS	Son of sevenless
T2D	Type 2 diabetes
TBE	Tris-borate-EDTA buffer
TBS	Tris- buffered saline
TBST-T	TBS with 0.1% Tween-20
TGF-α,β	Transforming growth factor-α,β
TNF-α	Tumour necrosis factor-α
VCAM-1	Vascular cell adhesion molecule-1
VEGF –A/B/C/D/E	Vascular endothelial growth factor–A/B/C/D/E
VEGFR-1/2/3	Vascular endothelial growth factor receptor-1/2/3
VHL	Von Hippel-Lindau
VSMCs	Vascular smooth muscle cells
vWf	von-Willebrand factor
Wb	Westernblotting

Chapter 1

Introduction

1.1. Angiogenesis

In mammals, the vascular system is the main supplier of oxygen and nutrients required for growth and survival. The formation and development of new blood vessels are essential during embryogenesis and throughout life for normal physiological processes such as wound healing, organ regeneration and in the female reproductive system during ovulation, menstruation and the formation of the placenta (Folkman, 1995). Abnormal growth of blood vessels has been linked to several disorders including proliferative diabetic retinopathy, cancer, rheumatoid arthritis, atherosclerosis, reviewed in (Folkman, 1971, Folkman, 1995, and Ross, 1999). Understanding the molecular mechanisms underpinning this process and how they are regulated would provide an ideal target to treat these diseases.

Formation and development of blood vessel networks differs throughout life. During embryogenesis, endothelial cell progenitors 'angioblasts', induced by fibroblast growth factor (FGF), coalesce to form some of the major vessels such as the aorta. This process is known as vasculogenesis (Flamme and Risau, 1992; Patz, 1978; Risau, 1997). The maturation and expansion of the primitive network is maintained by angiogenesis. Through this process, new vessels are formed from pre-existing ones by vessel splitting (termed sprouting angiogenesis), or develop by intussusception and vessel elongation, also known as (a.k.a), non-sprouting angiogenesis. These vessels undergo pruning and remodelling until a functional adult cardiovascular system is developed, reviewed in (Yancopoulos et al., 2000).

Angiogenesis is a multi-step process that is tightly co-ordinated by the fine balance between pro-angiogenic and anti-angiogenic factors. It involves endothelial cell division, degradation of the basement membrane and the surrounding extracellular matrix, endothelial cell migration and the formation of blood vessels. Angiogenesis is initiated in response to hypoxia through vascular relaxation which is mediated by nitric oxide (NO) (Carmeliet and Storkebaum, 2002; Folkman, 1997). Vascular endothelial factor (VEGF) activation of endothelial cells increases NO and the release of proteases such as, plasminogen activators and matrix metalloproteinases. These proteases loosen inter-endothelial contacts; promote detachment of smooth muscle cells as well as the degradation of the basement membrane of the existing vessels (Heymans et al., 1999). With the path cleared, the increased release of growth factors such as FGF-2, VEGF, and insulin-like growth factor-I (IGF-I) promotes endothelial cell proliferation and migration guided by the angiogenic stimuli. This step is followed by the formation of the blood vessel lumen (Risau, 1995). Platelet-derived growth factor (PDGF) released by endothelial cells, functions in a paracrine manner as a mitogen and chemoattractant for a variety of mesenchymal mural cells (pericytes in small vessels and smooth muscle cells in large vessels), which provide structural support, stability and maintain vessel integrity (Hirschi and D'Amore, 1997; Sims, 1986). Angiopoietins 1 & 2 and their receptors, Tie1 and 2, are required in the later stages of angiogenesis, as they play a major role in the communication between endothelial cells and mesenchyme in the surrounding microenvironment, hence, establishing stable cellular and biochemical interactions (Yancopoulos et al., 2000). Growth factors, cytokines and their receptors can function to regulate angiogenesis independently, collectively, or co-operate with environmental, or mechanical stimuli, to invoke a response to any physiological changes in the microenvironment (Pepper et al., 1998).

1.2. Vascular endothelial growth factor family

Since the breakthrough made by Folkman in the early 1970s implicating 'angiogenesis' in the development and spread of tumours (Folkman, 1971; Folkman et al., 1971), extensive work has been undertaken to identify the molecules and the mechanisms regulating this dynamic and complex process. These collective efforts led to the discovery of the VEGF family of growth factors (Ferrara and Henzel, 1989; Senger et al., 1990; Senger et al., 1983). These factors are secreted by several cell types as freely soluble or extracellular matrix (ECM), or cell membrane bound forms, and play a fundamental role in modulating angiogenesis in many species' in both physiology and pathology. The VEGF family members, placenta growth factor (PlGF) (Maglione et al., 1993; Maglione et al., 1991), VEGF-B (Olofsson et al., 1996), VEGF-C (Joukov et al., 1996; Lee et al., 1996), VEGF-D (Achen et al., 1998; Orlandini et al., 1996) and VEGF-E (Lyttle et al., 1994) are structurally related and belong to the platelet-derived growth factor PDGF/ VEGF super family. VEGF, a.k.a VEGF-A, has received the most attention and is a major regulator of both physiological and pathological angiogenesis.

1.2.1. Vascular endothelial growth factor

1.2.1.1. Structure and functional properties

The crystal structure of VEGF showed that it is comprised of two monomers organised in anti-parallel fashion to form a dimer with receptor–binding sites located at each pole (Keck et al., 1989; Muller et al., 1997). VEGF is a heparin-binding glycoprotein of 34-

42 kDa located at chromosome 6p21.3 and exists in at least seven homodimeric isoforms (121, 145, 148, 165, 183, 189 and 206) encoded by 8 exons separated by 7 introns (Houck et al., 1991; Tischer et al., 1991). Exons 1 to 5 and 8 are conserved in all isoforms except $VEGF_{148}$. VEGF isoforms differ by the presence or absence of sequences encoded by exons 6 and 7 through alternative mRNA splicing. The heparin-binding domain encoded by exon 6, determines the degree of binding to extracellular matrix (ECM). Therefore, $VEGF_{121}$ which lacks exon 6 does not bind to heparin or ECM and is freely soluble (Houck et al., 1992). $VEGF_{165}$, the predominant isoform, has a moderate heparin binding ability, whereas $VEGF_{145}$, $VEGF_{189}$ and $VEGF_{206}$ are the least diffusible and hence are completely sequestered in the ECM (Park et al., 1993). Recently, a new group of VEGF-A isoforms generated by alternative splicing at a more distal splice site in exon 8 have been identified. These isoforms are the same length as the classic VEGF isoforms but differ in their C-terminal 6 amino acids. They were assigned with the name VEGF xxx b, where xxx denoting the amino-acid number of the mature protein. Early studies on this family indicated that VEGF xxx b inhibits most of the pro-angiogenic functions of VEGF (Bates et al., 2002), a feature that could be exploited for anti-angiogenic therapeutic purposes. More studies are required to fully determine the precise biological function of this family.

1.2.1.2. Regulation of VEGF gene expression

The hypoxia inducible factor-1 (HIF-1) transcription factor is the major regulator of VEGF expression in various pathologies (Dor et al., 2001; Safran and Kaelin, 2003). The HIF family plays a role in the adaptive response to hypoxia in embryo development and the adult (Berra et al., 2006; Zhou et al., 2006). HIF-1 is a basic heterodimeric

helix-loop-helix protein consisting of two subunits (HIF-1α, HIF-1β) that binds an enhancer sequence on the 5'-promoter of the VEGF gene resulting in the up-regulation and enhanced stability of the VEGF mRNA (Liu et al., 1995; Wang and Semenza, 1995). In some cancers, such as cerebellar hemangioblastomas and human renal carcinomas, inactivating mutations in the von Hippel-Lindau (VHL) tumour suppressor gene were reported to be the cause of the high VEGF expression in these tumours (Lonser et al., 2003). VHL targets the degradation of HIF subunits; hence, the loss of VHL results in increased levels of HIF1α (Ivan et al., 2001; Jaakkola et al., 2001). Zundel et al (2000) showed that the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) signalling pathway is involved in the regulation of HIF-mediated responses outside the context of the hypoxic microenvironment. The sustained activity of PI3K/Akt axis resulting from a mutation in phosphatase and tensin homolog on chromosome 10 (PTEN) acting upstream of this pathway led to increased activity of HIF-1 and enhanced VEGF production. Several growth factors i.e. IGF-I also up-regulate VEGF mRNA expression in a paracrine or autocrine manner independently of hypoxia in the microenvironment (Warren et al., 1996). Pro-inflammatory cytokines such as interlukin-1α (IL-1α) in human synovial fibroblasts, and interleukin-6 (IL-6) in tumour cell lines have also been shown to stimulate VEGF production in a wide range of inflammatory disorders (Ben-Av et al., 1995).

1.2.2. Biological functions of VEGF

1.2.2.1. Endothelial cell mitogenesis, angiogenesis and survival

The loss of a single VEGF allele during embryonic development is lethal. The heterozygous ($VEGF^{+/-}$) mouse embryos die between day 11 and 12, exhibit significant defects in the vasculature of several organs and tissues such as placenta, the nervous system, and markedly reduced number of nucleated red blood cells within blood islands in the yolk sac (Carmeliet et al., 1996; Ferrara et al., 1996). VEGF over-expression in mice hearts results in severe abnormalities in heart development and lethality at embryonic days 12.5 and 14 (Miquerol et al., 2000). This demonstrates the importance of tight regulation of VEGF levels during development. The selective knockout of the VEGF $^{120/120}$ isoform is also lethal, as most mice die *in utero* or soon after birth (Carmeliet et al., 1999). It has been established that VEGF promotes the growth and proliferation of endothelial cells, and promotes angiogenesis by inducing cell migration, tube formation and sprouting from aortic rings *in vitro* (Nicosia et al., 1994; Pepper et al., 1992). VEGF has also been shown to enhance lymph angiogenesis *in vivo* (Nagy et al., 2002)

VEGF plays an important role in endothelial cell survival. VEGF can maintain the viability of endothelial cells in the absence of serum by activating the PI3K/Akt survival signalling pathway (Fujio and Walsh, 1999), leading to induction of pro-survival proteins such as Bcl-2 (Gerber et al., 1998a) and the suppression of proteins that promote cell apoptosis and cell cycle arrest such as forkhead boxO1 (FOXO1) and forkhead boxO3a (FOXO-3a) in cultured endothelial cells (Potente et al., 2005).

1.2.2.2. Enhancement of vascular permeability and haemodynamic effects

VEGF was discovered due to its ability to induce vascular leakage (Dvorak et al., 1995; Senger et al., 1983). NO is an essential mediator of this process (Morbidelli et al., 1996; Parenti et al., 1998). Fukumura et al, 2001 showed that processes such as angiogenesis, vessel diameter, blood flow rate and vascular permeability are all NO-dependent and proportionate to NO levels produced. These processes are impaired in endothelial nitric oxide synthase (eNOS) knockout mice. VEGF is implicated in monocyte chemotaxis, which plays an important role in VEGF-mediated promotion of angiogenic processes such as collateral growth and arteriogeneis (Clauss et al., 1990). VEGF administration to adult mice blocks the development of dendritic cells and this was shown to promote tumour evasion of the host's immune system. VEGF also plays a role in vascular smooth muscle cell proliferation and migration (Bhardwaj et al., 2005). Interestingly, VEGF was also shown to mediate the proliferation and migration of blood cells in Drosophila (Cho et al., 2002).

1.3. Vascular endothelial growth factor receptors

The receptors for VEGF family members are three high affinity receptor tyrosine kinases (RTKs), VEGFR-1, a.k.a. Fms-like tyrosine kinase-1 (Flt-1), VEGFR-2 a.k.a. kinase insert domain receptor (KDR) and VEGFR-3 a.k.a. Fms-like tyrosine kinase-4 (Flt-4), and two accessory, non-enzymatic receptors, neuropilin-1 and -2 (NP-1) and (NP-2). As shown in Figure 1.1, VEGF-A binds VEGFR-1 and VEGFR-2, whereas PlGF and VEGF-B only binds VEGFR-1. Both VEGF-C and VEGF-D bind VEGFR-2 and VEGFR-3 (Karkkainen et al., 2002; Pajusola et al., 1992). NP-1 and NP-2 are

members of the semaphorin family and implicated in neuronal development and angiogenesis (Klagsbrun et al., 2002; Neufeld et al., 2002). NP-1 binds $VEGF_{165}$, VEGF-B and PlGF, but fails to bind $VEGF_{121}$; whereas, NP-2 binds $VEGF_{165}$, VEGF-C and PlGF.

VEGFR-1 and VEGFR-2 were first identified on vascular endothelial cells *in vitro* (Plouet and Moukadiri, 1990; Vaisman et al., 1990) and *in vivo* (Jakeman et al., 1993; Jakeman et al., 1992). These receptors are not exclusively expressed on endothelial cells, but are also present on other cell types such as bone marrow-derived cells, monocytes (Shen et al., 1993), vascular smooth muscle cells (VSMCs) (Ishida et al., 2001) and cancer cells (Skobe et al., 1997). VEGFR-1 and VEGFR-2 are highly related RTKs that consist of a seven immunoglobulin IgG-like domains in the extracellular domain, a single transmembrane region, and tyrosine kinase domain that is interrupted by a 70 amino-acid kinase–insert domain, and a C-terminal tail (Matthews et al., 1991; Shibuya et al., 1990; Terman et al., 1991). Mutation studies have shown that the second and third IgG-like domains constitute the ligand-binding domains, whereas the fourth IgG-like domain is responsible for dimerisation (Davis-Smyth et al., 1998; Fuh et al., 1998; Shinkai et al., 1998). Differences in the VEGFR domain structure underpins the variance in their biological activities. The VEGF family members show different affinities for neuropilins (NPs) and heparin sulfate proteoglycans, which are also implicated in the modulation of VEGFR-functions.

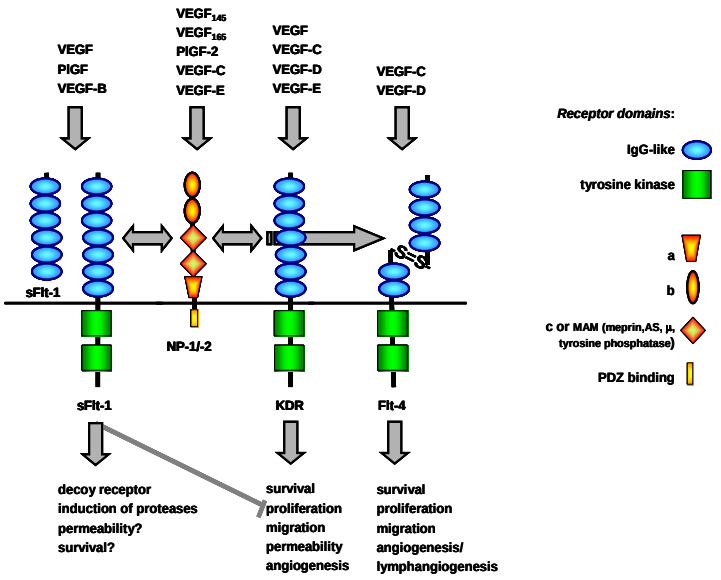

Figure 1.1: The interactions of the VEGFs with their high-affinity tyrosine kinase receptors Flt-1, s-Flt-1, KDR, Flt-4, and accessory receptors, neuropilin-1 and –2 (NP-1/-2). *VEGFR-1/Flt-1, VEGFR-2/KDR and VEGFR-3/Flt-4 possess 7 immunoglobulin-like loops in the extracellular domain and an intracellular split tyrosine kinase domain and bind VEGFs selectively. NP-1 and NP-2 bind VEGF and PlGF in an isoform-specific manner and interact with Flt-1, KDR and Flt-4 to modulate their activity. Signalling diversity is increased further by the expression of VEGF heterodimers and formation of heterodimeric receptor complexes (kindly provided by Dr PW Hewett).*

1.3.1. Vascular endothelial growth factor receptor-1 (VEGFR-1)

VEGFR-1 is 180 kDa glycoprotein and product of the human *flt-1* gene which is located on chromosome 13q12 (de Vries et al., 1992; Rosnet et al., 1991). VEGFR-1 is predominantly expressed on endothelial cells, but it is also detected on placental trophoblasts, osteoblasts, monocytes, macrophages, renal mesangial, thyroid cells and in some hematopoietic stem cells (Ahmed et al., 1995; Barleon et al., 1996; Susarla et al., 2007; Takahashi et al., 1995). VEGFR-1 binds VEGF-A, VEGF-B and PlGF with high affinity. This receptor is essential for development during embryogenesis as mice lacking VEGFR-1, as shown in Figure 1.2, die at E8.5 as a result of overgrowth of endothelial cells and vasculature (Fong et al., 1995), however, mice expressing

VEGFR-1 lacking the tyrosine kinase domain survive, suggesting that VEGFR-1 signalling is not essential, and acts as decoy receptor for VEGF during development (Hiratsuka et al., 1998).

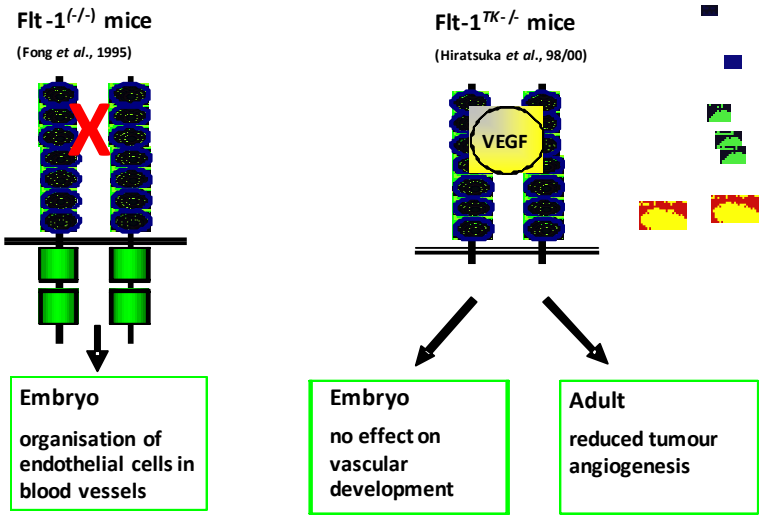

***Figure 1.2. Role of VEGFR-1 in the regulation of angiogenesis**. VEGFR-1 has a very low tyrosine activity. Mouse embryos lacking VEGFR-1 die as a result of vascular defects, whereas, mice expressing VEGFR-1 without the tyrosine kinase domain survive, suggesting that VEGFR-1 signalling is not essential, and acts as a decoy receptor for VEGF during development. However, the truncation of tyrosine kinase domains or down-regulation of VEGFR-1 suppresses tumour growth and abrogates VEGF-induced angiogenesis (kindly provided by Dr PW Hewett).*

VEGFR-1 has a higher affinity (over 10-fold) for VEGF-A than VEGFR-2 does, but is only weakly phosphorylated generating a very weak kinase signal, and is unable to elicit a mitogenic response in endothelial cells (Waltenberger et al., 1994), indicating that VEGFR-1 may not primarily function as a signalling receptor, but rather as a decoy receptor that negatively regulates VEGF activities on the endothelium by sequestration. It later became clear that VEGFR-1 is also capable of transmitting signals in its own right as well as forming heterodimers with VEGFR-2 that have strong signalling properties (Autiero et al., 2003a; Huang et al., 2001). VEGFR-1 activation by PlGF

promotes angiogenesis either independently or through intracellular crosstalk with VEGFR-2 (Autiero et al., 2003a). Moreover, VEGFR-1 is essential for VEGF as well as PlGF-mediated monocyte chemotaxis and in the recruitment and survival of bone marrow-derived progenitor cells (Barleon et al., 1996; Hattori et al., 2002). Recently, a role for VEGFR-1 in the regulation of metabolism was identified. VEGFR-1 binding to VEGF-B was shown to mediate the induction of fatty acid up-take in endothelial cells (Hagberg et al., 2010). Growth factor binding to the extracellular domain of an RTK triggers homo-dimerisation, or heterodimerisation with other adjacent RTKs, leading to rapid activation of the receptor and auto-phosphorylation of multiple specific intracellular tyrosine residues in the cytoplasmic kinase domain. VEGFR-1 is autophosphorylated at Y 1169, 1213, 1242, 1327 (Cunningham et al., 1995; Ito et al., 2001; Sawano et al., 1997). PlGF binding to VEGFR-1 activates PI3K/Akt and promotes survival in endothelial cells and monocytes (Cai et al., 2003b), and NO production by inducing the phosphorylation of eNOS on serine 1177 (Ahmad et al., 2006). Insights into the role of VEGFR-1 *in vivo* have been shown by inhibiting its function. The inhibition of VEGFR-1 using a specific VEGFR-1 antibody suppressed neovascularisation in tumours, inflammation in arthritis and plaque formation and rupture in atherosclerosis in mice (Luttun et al., 2002).

A soluble form of VEGFR-1 (sVEGFR-1; a.k.a sFlt-1) results from alternative splicing of VEGFR-1 gene and consists only of the extracellular domain of VEGFR-1. As shown in Figure 1.3, s-VEGFR-1 has the ability to A) inhibit the actions of VEGF and PlGF by preventing their interaction with their respective RTKs. It can also interact with itself and VEGFR-2 forming homo/heterodimers formation and transphosphorylation/activation (B and C) (Kendall et al., 1996). In humans and mice,

s-VEGFR-1 has been linked to preeclampsia and inhibition of wound healing (Ahmad and Ahmed, 2004; Carmeliet et al., 2001; Maynard et al., 2003). To sum up, VEGFR-1 may act as a positive or a negative regulator of angiogenesis depending on the circumstances.

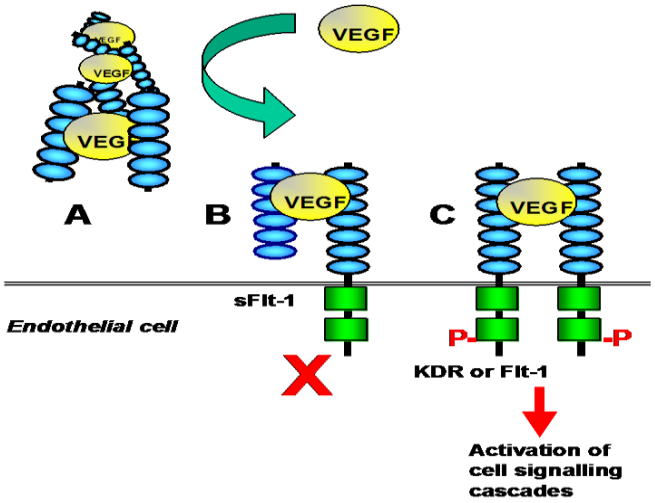

***Figure 1.3**: **s-VEGFR-1/sFlt-1 regulates VEGF availability and activity**: **A** It acts as sink, sequestering its ligands, VEGF, PlGF and VEGF-B preventing binding to Flt and KDR. **B** sFlt-1 also acts in a dominant-negative manner to block the formation of Flt-1 and KDR homo- and heterodimers and so prevent **C** their transphorylation/activation (Kendall et al., 1996(Hewett, 2006)*

1.3.2. Vascular endothelial growth factor receptor-2 (VEGFR-2)

VEGFR-2 is a.k.a. KDR in human (Terman et al., 1992b) and fetal liver kinase receptor (Flk-1) in mice. Human VEGFR-2 was first cloned from a human endothelial cell cDNA library (Terman et al., 1991; Terman et al., 1992a). VEGFR-2 is primarily expressed on endothelial cells, but is also detected in hematopoietic stem cells, megakaryocytes, retinal progenitor cells and tumour cells (Katoh et al., 1995; Thieme et al., 1995; Yang and Cepko, 1996). The expression of VEGFR-2 is first detected at E 7.5 in mesodermal cells (Kaipainen et al., 1993; Shalaby et al., 1995). VEGFR-2

knockout mice die *in utero* between 8.5 and 9.5 days due to impaired development of haematopoietic, endothelial cells and failure to develop blood islands and organised blood vessels (Shalaby et al., 1995)

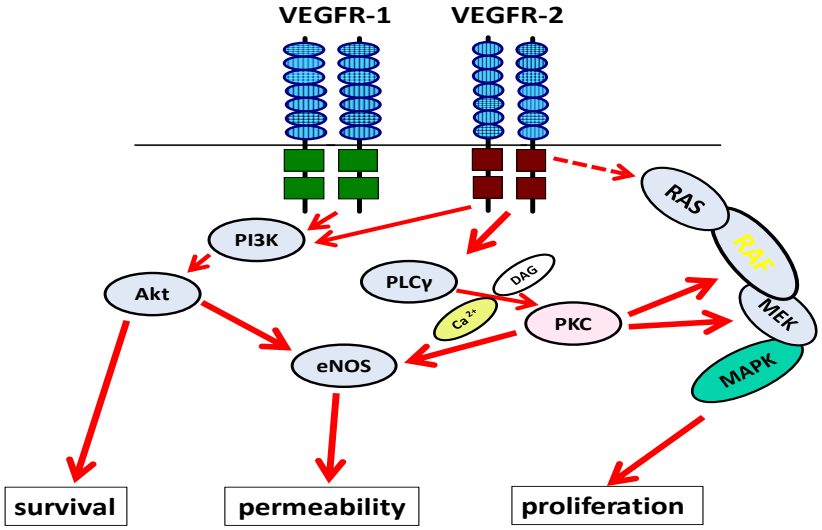

***Figure 1.4. VEGFR-1 and VEGFR-2 signal transduction pathways in endothelial cells**. In endothelial cells growth factor activation of the PI3K/Akt promotes cell survival and nitric oxide (NO) generation via phosphorylation of eNOS. The activation of the MAPK/ERK signalling cascade, via PLCγ and PKC controls cell proliferation via VEGFR-2. VEGFR-2–mediated activation of PLCγ of PKC can also promote NO production via phosphorylation of eNOS. eNOS, endothelial nitric oxide synthase; MAPK, mitogen-activated protein kinase; MEK, MAPK; PI3K, phosphatidylinositol 3′ kinase; PKC, protein kinase C; PLCγ, phospholipase Cγ, DAG, diacylglycerol, Ca^{2+},calcium.*

Although VEGFR-2 binding affinity to VEGF is lower than that of VEGFR-1, the majority of VEGF biological functions, including mitogenic, angiogenic, and permeability-enhancing effects in physiological and pathological angiogenesis, are elicited through VEGFR-2.

VEGFR-2 is highly expressed in tumour vasculature compared to normal vasculature (Plate et al., 1994). Upon stimulation with VEGF, VEGFR-2 undergoes dimerisation and auto-phosphorylation of its tyrosines in the intracellular domain in a ligand-

dependent manner. Tyrosine residues at 951,1054,1059,1175 and 1214 are phosphorylated in human VEGFR-2 (Shibuya and Claesson-Welsh, 2006). Y1175 and Y1214 are the two major VEGF–dependent auto-phosphorylation sites in VEGFR-2. Auto-phosphorylation of Y1175 is essential for VEGF-dependent endothelial cell proliferation (Takahashi et al., 2001). As shown in Figure 1.4, VEGF induces the phosphorylation and activation of phospholipase Cγ (PLCγ) resulting in diacylglycerol formation and thus the activation of protein kinase C (PKC) (Takahashi et al., 2001). Unlike other conventional RTKs, VEGFR2-mediated mitogen-activated-protein kinase (MAPK) activation is not thought to be coupled to Ras. Instead, MAPK is stimulated but via phospholipase Cγ (PLCγ) and PKC activation of Raf (Takahashi et al., 1999). In this study, Ras is poorly activated by VEGFR-2 activation, and p42/44 MAPK stimulation is Raf-mediated via PKC following PLCγ activation in endothelial cells of the rat liver. Control of proliferation and growth by other growth factors is mediated by the Ras/MAP-kinase pathway. Binding of growth factors e.g. EGF to their respective RTKs causes the recruitment of SH2 domain-containing adaptor proteins such as Grb2, the formation of this complex triggers the translocation of the son of sevenless (SOS) into close proximity of Ras plasma membrane, this promotes the conversion of the inactive GDP-associated with Ras into active GTP. This initiates a phosphorylation cascade involving Raf, mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase kinase (MEK) and MAPK (Stokoe et al., 1994). Endothelial cell survival and chemotaxis is mediated via the PI3K/Akt pathway (Byzova et al., 2000; Gerber et al., 1998b). Other pathways activated by VEGF via VEGFR-2 include Ras GTPase activating protein (Guo et al., 1995).

1.4. Placental growth factor (PlGF)

1.4.1. Structure and functional properties

PlGF was first discovered by Persico et al., (1991) in a human placenta cDNA library; hence its name. It is a homodimeric glycoprotein that shares 42% amino acid sequence identity with VEGF and has a very similar tertiary structure (Iyer et al., 2001; Maglione et al., 1991). The human PlGF gene is located on chromosome 14 and encodes seven exons. In humans, alternative mRNA splicing yields four PlGF (1-4) isoforms of 131, 152, 203 and 224 amino-acid chain length. Whereas, the mouse only expresses PlGF-2. PlGF has been detected in many human tissues, including, breast (Parr et al., 2005), stomach (Chen et al., 2004), prostate (Matsumoto et al., 2003a), heart (Iwama et al., 2006), lung (Persico et al., 1999), retina (Feeney et al., 2003), thyroid (Viglietto et al., 1995), and skin (Failla et al., 2000; Odorisio et al., 2006). PlGF is expressed by several cell types including, endothelial cells, thyroid epithelial cells, VSMC, pro-inflammatory cells (i,e monocytes and macrophages), bone marrow cells, neurons and keratinocytes (Failla et al., 2000) and trophoblasts (Khaliq et al., 1996; Shore et al., 1997; Vuorela et al., 1997), in particular when these cells are stressed, or activated (Beck et al., 2002; Iyer and Acharya, 2002; Luttun et al., 2002). Although tumours respond to PlGF and some tumour cells such as choriocarcinoma cells produce it; whether tumour cells actually secrete significant amounts of PlGF is controversial as conditioned-medium from various cancer cell lines were devoid of PlGF (PWH unpublished data). This suggests that the source of PlGF detected in certain cancers is produced by the stromal cells, such as endothelial and inflammatory cells.

PlGF-1 is a dimeric protein with a molecular weight of 46 kDa and is composed of 131 amino acid residues per monomer, whereas PlGF-2 and 4 contain additional 21 basic residues that confer ability to bind negatively charged heparan sulfate proteoglycans. PlGF-3 is structurally similar to PlGF-4 but lacks the heparin binding domain (Yang et al., 2003). Little is known about PlGF isoforms and the differences in their function. PlGF can naturally form heterodimers with VEGF (DiSalvo et al., 1995). VEGF:PlGF heterodimers were detected in tumour cell supernatants and their activity has controversially been reported to be less effective than $VEGF_{165}$ homodimers (Cao et al., 1996; Eriksson et al., 2002; Xu et al., 2006). These heterodimers promote the formation of VEGFR1:VEGFR2 heterodimers to which they preferentially bind (Autiero et al., 2003a), they also bind VEGFR1:VEGFR1, but not VEGFR-2:VEGFR-2 (Eriksson et al., 2002)

Although the role of PlGF in pathology has been extensively studied, there is very limited information on its transcriptional regulation, in particular, in endothelial cells. In preeclampsia, a pregnancy disorder characterised by placental hypoxia and deficiency in vascularisation. There has been a reported to be a decrease in PlGF secretion by placental trophoblasts in the serum of preeclamptic women (Levine et al., 2006). In recent years several transcription factors binding sites have been identified in the PlGF promoter. The metal-responsive transcription factor (MTF-1), has been implicated in the hypoxia stimulated up-regulation of PlGF expression in fibroblasts and human choriocarcinoma cell lines (Green et al., 2001; Nishimoto et al., 2009). Promoter deletion analysis demonstrated the existence of multiple MTF-1 binding sites in the PlGF promoter which have been shown to be important for the hypoxic regulation of PlGF in the BeWo choriocarcinoma cell line. The binding of MTF-1 to

the PlGF promoter has been shown using ChIP analysis (Nishimoto et al., 2009). The 5'UTR of the PlGF gene contains a number of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) sites which are reported to be of significance in the regulation of PlGF gene activity in response to inflammatory cytokines in fibroblasts (Cramer et al., 2005). PlGF is also reported to be selectively switched on for a short period during the development in the embryonic kidney by the epithelium-specific BF-2 (FoxD1) transcription factor in mesenchymal cells (Zhang et al., 2003). In a very recent report, two functional cAMP responsive elements (CRE) in the PlGF promoter have been identified which bind the CRE binding protein, CREB, and contribute to cAMP mediate regulation of gene expression (Depoix et al., 2011).

1.4.2. PlGF and receptor interactions

Unlike VEGF, PlGF binds only to VEGFR-1 and NP-1 and NP-2 (Migdal et al., 1998; Persico et al., 1999). The binding of PlGF to VEGFR-1 has been shown to lead to the phosphorylation of alternative tyrosine residues in VEGFR-1 and gene activation distinct from those elicited by VEGF (Autiero et al., 2003b). In addition, as shown Figure 1.5, PlGF can induce signals though VEGFR-1 independently of VEGF (Ahmad et al., 2006; Cai et al., 2003b). As shown in Figure 1.6, PlGF binding to VEGFR-1 and s-VEGFR-1 prevents VEGF from binding to or displaces already bound VEGF from VEGFR-1, thereby, increasing the concentration of VEGF available to activate VEGFR-2 and enhance VEGF-driven angiogenesis (displacement theory) (Park et al., 1994). Furthermore, the binding of PlGF to VEGFR-1 triggers VEGFR-1 and VEGFR-2 molecular crosstalk, consequently enhancing VEGFR-2 signalling and VEGF-driven angiogenesis, as shown in Figure 1.7 (Autiero et al., 2003a)

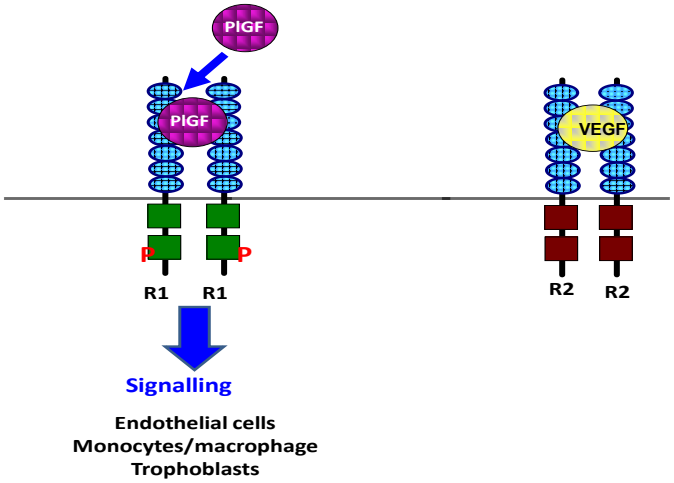

***Figure 1.5. PlGF enhances angiogenesis directly**. PlGF can elicit its biological functions through its direct interaction/activation of VEGFR-1 in endothelial cells and VEGFR-1-expressing cells. PlGF was shown to induce the phosphorylation of tyrosines distinct from the ones induced by VEGF in VEGFR-1, indicating that PlGF, in its own right, transmits angiogenic signals via VEGFR-1 distinct from those of VEGF (Autiero et al., 2003a) (kindly provided by Dr PW Hewett).*

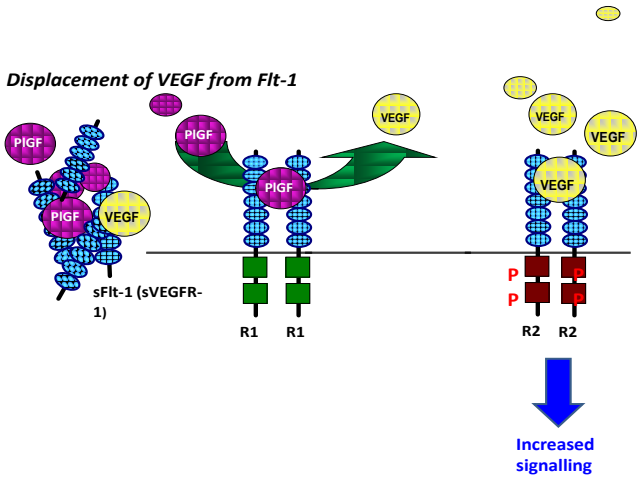

***Figure 1.6. PlGF enhances VEGF-driven angiogenesis**. By preferentially binding to VEGFR-1, PlGF displaces VEGF from VEGFR-1, thereby making more VEGF available to bind and induce VEGFR-2 signalling (kindly provided by Dr PW Hewett).*

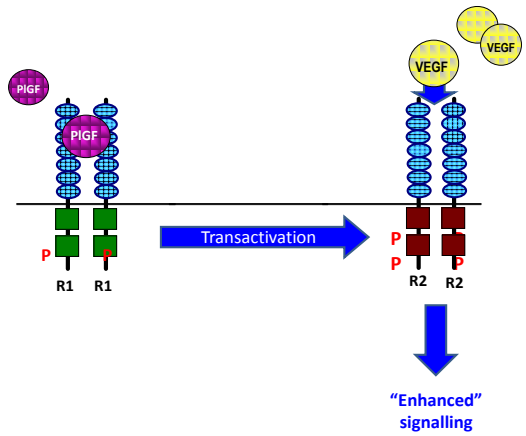

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***Figure 1.7. PlGF can also co-operate with VEGF to promote angiogenesis**. Activation of VEGFR-1 by PlGF triggers intermolecular cross-talk between VEGFR-1 and VEGFR-2, thereby inducing the VEGF angiogenic VEGR-2-mediated signals (kindly provided by Dr PW Hewett).*

1.4.3. PlGF biological functions

PlGF knockout mice are viable and fertile without major vascular defects. This indicates a minor role for PlGF during embryonic development (Carmeliet et al., 2001). PlGF plays an essential role in angiogenesis-associated pathologies throughout adulthood and induces a wide range of biological effects *in vivo* and *in vitro* through various cell types. PlGF enhances angiogenesis by acting directly or through pro-angiogenic mediators (Cai et al., 2003b; Khaliq et al., 1996), stimulates migration and survival of endothelial cells (Adini et al., 2002; Cai et al., 2003b; Fischer et al., 2007; Ziche et al., 1997), induces vasodilatation and stimulates collateral vessel growth (Bellik et al., 2005; Yonekura et al., 1999). PlGF can also directly contribute to the maturation of newly formed blood vessels by promoting the recruitment and

mobilisation of VEGFR-1-expressing endothelial progenitor cells (EPC) and stem cells (Hattori et al., 2002; Luttun et al., 2002; Pipp et al., 2003).

1.5. The role of PlGF in pathology

1.5.1. PlGF and VEGFR-1 in inflammatory disorders

Atherosclerosis and rheumatoid arthritis (RA) are chronic inflammatory disorders characterized by angiogenesis. Adenoviral delivery of PlGF-2 into apolipoprotein-E-deficient ($ApoE^{-/-}$) mice increased intimal thickening and macrophage accumulation compared with $ApoE^{-/-}$ and $PlGF^{-/-}$ mice, suggesting a role for PlGF in the promotion of atherosclerosis (Khurana et al., 2005). In addition, the inhibition of VEGFR-1 significantly reduced the size of atherosclerotic lesions in $ApoE^{-/-}$ mice. These mice are susceptible to developing atherosclerosis due to accumulation of lipids and increased inflammation in the proximal aorta when fed high fat diet (Luttun et al., 2002). The blockade of VEGFR-1 had a dramatic effect on the cellular content of the plaques, as up to 40% of macrophage transmigration was reduced, whereas anti-VEGFR-2 had no effect, indicating that the increase in the atherosclerotic plaque size is attributed to inflammation rather than angiogenesis, thus, it was concluded that the suppression of inflammatory cell infiltration by blocking VEGFR-1 confers atheroprotection. In addition, the elevation of PlGF expression in patients with atherosclerosis is correlated with inflammation, vascularisation and plaque instability (Pilarczyk et al., 2008). In rheumatoid arthritis (RA) growth factors, cytokines, chemokines as well as adhesion molecules and matrix-degrading proteases have been detected in the inflamed synovium (Bodolay et al., 2002). Synoviocyte proliferation,

infiltration of inflammatory cells and cartilage destruction are all hallmarks of this disease (Lee and Weinblatt, 2001; Szekanecz and Koch, 2007; Weber and De Bandt, 2000). In an animal model mimicking human arthritis (Courtenay et al., 1980), VEGFR-1 and PlGF were detected on inflammatory and endothelial cells in the inflamed synovium in the affected joints. The inhibition of VEGFR-1 led to a 60% decrease in the incidence of the joint disease, suppressed the development of clinical symptoms by up to 85% and protected against bone destruction compared with anti-VEGFR-2 treatment, which was ineffective. All these effects can be explained by the fact that inhibition of VEGFR-1, which is expressed by inflammatory cells such as monocytes and macrophages, blocked PlGF-stimulated inflammatory cell infiltration and activation of leucocytes. Taken together, the failure of anti-VEGFR-2 to block atherosclerosis and arthritis independently of angiogenesis indicates that it is PlGF acting via VEGFR-1 to promote inflammation (Luttun et al., 2002). Furthermore, anti-VEGFR-1 blocked the recruitment of bone marrow-derived cells into atherosclerotic plaques and arthritic joints (Luttun et al., 2002). The lack of leukocyte accumulation was partly due to the inhibition of myeloid cell transmigration, differentiation and reduced mobilisation from the bone marrow into the peripheral blood. Also, anti-VEGFR-1 suppressed the mobilization of hematopoietic progenitors into the peripheral blood by 75% (Luttun et al., 2002). PlGF has also been reported to drive VEGFR-1-dependent mobilization of haematopoietic stem and progenitor cells to reconstitute haematopoiesis (Hattori et al., 2002; Heissig et al., 2002).

1.5.2. The Role of PlGF in Cancer

That tumours grown in PlGF knockout mice were small and avascular compared with tumours implanted in wild-type counterparts, was the first evidence presented linking PlGF to cancer promotion (Luttun et al., 2002). PlGF has been implicated in the promotion of tumour growth by enhancing angiogenesis and conferring resistance to anti-tumour treatment (Escudero-Esparza et al., 2009; Fischer et al., 2007; Luttun et al., 2002; Zhang et al., 2005), but, there are conflicting reports regarding the types of tumours that express PlGF. PlGF mRNA and protein levels are increased in gastric cancer (Chen et al., 2004), correlate with disease progression and survival in colorectal cancer (Wei et al., 2005), tumour stage in lung cancer (Zhang et al., 2005) and recurrence and mortality in breast cancer (Parr et al., 2005). In renal cancer, PlGF plasma levels correlate with tumour stage and survival (Matsumoto et al., 2003b). In contrast, PlGF is not detectable in some cancers, i.e. ovarian cancer (Sowter et al., 1997) and is reduced in thyroid cancer (Viglietto et al., 1995). In recent reports, PlGF was proposed as a potential prognostic marker for certain types of cancers (Zhang et al., 2005).

The development of anti-PlGF antibody was proposed as a therapy for some, but not all tumours. Previous studies showed that anti-PlGF antibody inhibited tumour neovascularisation and the pre-existing tumour vasculature, and suppressed inflammation through it is ability to block the recruitment of inflammatory cells to tumour microenvironment (Fischer et al., 2007; Van de Veire et al., 2010). A key feature of anti-PlGF therapy is its safety profile, as it does not affect healthy vessels and it was claimed that it did not cause side effects associated with anti-VEGF therapy

(hypoxia). These findings have recently been contested by Bais et al (2010) who found that anti-PlGF therapy had no effect on 15 types of tumours tested. Furthermore, anti-PlGF did not have an additive effect to anti-VEGF therapy (Fischer et al., 2007). In a very recent report host-produced histidine-rich glycoprotein (HRG) was shown to inhibit tumour growth and metastasis by induction of macrophage polarisation and vessel normalisation through down-regulation of PlGF (Rolny et al., 2011).

Increased levels of VEGFR-1 are reported in various types of cancers by immunohistochemistry in breast cancer, oesophageal cancer and prostate cancer (Fragoso et al., 2008; Jackson et al., 2002; Kato et al., 2002). The contribution of PlGF to tumour growth and progression is primarily via its ability to directly recruit and promote the proliferation and migration of VEGFR-1-expressing tumour cells (Fischer et al., 2007), and suppression of the immune system by blocking the maturation of myeloid dendritic cells (Dikov et al., 2005). In addition, PlGF can induce the growth of tumour blood vessels and influence the tumour microenvironment. PlGF also recruits VEGFR-1-expressing bone marrow stem cells which contribute to the growing sprouts of blood vessels by direct incorporation into the vessel wall, or indirectly, by secreting pro-angiogenic growth factors (Hattori et al., 2002; Luttun et al., 2002; Pipp et al., 2003). PlGF stimulates the recruitment of pericytes, smooth muscle cells and inflammatory cells such as macrophages to tumour sites (Adini et al., 2002; Fischer et al., 2008). The angiogenic switch during VEGFR-1 inhibition results in direct PlGF up-regulation in mice and cancer patients (Casanovas et al., 2005; Fischer et al., 2007; Willett et al., 2004). Taken together, PlGF and its receptor may provide an excellent target for therapies aimed at treating cancer, and clinical trials are ongoing for the proposed anti-PlGF therapy. The findings of these trials will ultimately help address

the unanswered questions about the role of PlGF in the promotion/progression of cancer in humans.

1.5.3. The role of PlGF in diabetic complications.

Abnormal endothelium function, a.k.a. endothelial dysfunction, resulting in loss of vascular integrity is a hallmark of diseases characterised by hyperglycaemia such as diabetes mellitus (Song et al., 2007). Clinical trials aimed at rigorous control of glucose levels showed reduced the risk of diabetes-associated complications such as retinopathy, neuropathy, nephropathy and atherosclerosis, thus providing evidence linking hyperglycaemia to the progression or the exacerbation, of micro- and macrovascular disorders (Nathan et al., 2003).

Vascular and neural systems in the human body are interlinked, and microvascular impairment in diabetes has been associated with the progression of neural dysfunction. It was demonstrated that endothelial dysfunction characterised by reduced NO production was associated with the development of foot ulceration in diabetic patients (Veves et al., 1998). Diabetic retinopathy is the major cause of impairment of visual function, leading to blindness (Engerman and Kern, 1995). Pathological retinal neovascularisation occurs in up to 20% of diabetic patients, resulting from an imbalance between pro- and anti-angiogenic factors (Adamis et al., 1994; Aiello et al., 1994; Arfken et al., 1998). PlGF is elevated during development of proliferative diabetic retinopathy (Khaliq et al., 1998; Mitamura et al., 2002) and PlGF immunoreactivity is localised in the vitreous of diabetic patients, but was absent in healthy controls (Khaliq

et al., 1998). In diabetic retinopathy, PlGF mRNA was up-regulated in the retina (Spirin et al., 1999), and PlGF deficiency prevented the development of experimental choroidal neovascularisation (Rakic et al., 2003). Furthermore, Holborn et al (2006) showed that retinal pigment epithelium cells (RPE) express both, PlGF, VEGFR-1 and neuropilins, which were found to regulate the chemotaxis of RPE cells. PlGF is a major factor in the induction of RPE-blood–retinal barrier disruption in a VEGFR-1-dependent manner in diabetic retinopathy (Miyamoto et al., 2007).

Inflammation is implicated in the molecular pathophysiology of diabetic retinopathy (Adamis, 2002), vascular endothelial dysfunction and endothelial cell death in diabetes (Mizutani et al., 1996; Song et al., 2007). In these inflammatory disorders the PI3K/Akt signalling pathway, which functions as a link between the forkhead transcription factors, insulin and IGF-1 receptors is significantly suppressed (Kops et al., 1999; Song et al., 2007).

1.6. Insulin/IGF-I metabolic signalling pathway

1.6.1. Insulin/IGF-I receptors

The Insulin and insulin-like growth factor-I (IGF-I) receptor tyrosine kinases (RTKs) are tetrameric proteins consisting of two glycosylated and crosslinked alpha and beta subunits. The α chains are extracellular and bind their respective ligands (insulin or IGF-I) while the β subunits span the membrane and are responsible for intracellular signal transduction upon ligand stimulation (LeRoith et al., 1995; Seino et al., 1989).

There is a 60% amino-acid homology between IGF-1R and the insulin receptor (IR), and under certain physiological conditions they form functional heterodimers (Butler and LeRoith, 2001). Both receptors are expressed on micro- and macrovascular endothelial cells (Jialal et al., 1985). In human endothelial cells the expression ratio of IGF-IR to IR expression on the cell surface is 10 to 1, approximately 400,000 IGF-IR to 40,000 IR per cell (Zeng and Quon, 1996). IGFR-1:IR heterodimer affinity is low for insulin (Nitert et al., 2005). The physiological concentration of insulin (100-500 pM) activates IR signalling, but fails to stimulate IGF-1R and IGF-1R:IR heterodimer signalling (Li et al., 2005; Zeng and Quon, 1996). The concentration of insulin required to produce the same amount of NO in HUVEC is twice of that of IGF-1 (Zeng and Quon, 1996). Inhibitory insulin and IGF-1 mutant receptors have been implicated in the development of diabetes, insulin resistance and atherosclerosis (Saltiel and Kahn, 2001). Insulin/IGF-I receptor signalling activity is limited by tyrosine phosphatases (PTPases) which dephosphorylate key tyrosine residues (Elchebly et al., 1999). Signalling is also inhibited by suppressor of cytokine signalling-1 and 3 (SOCS1 and SOCS3) (Emanuelli et al., 2001; Ueki et al., 2004). SOCS1 and SOCS3 expression is increased in insulin-resistant states and obesity suggesting that these inhibitors might play role in the pathology of diabetes. The insulin-like growth factor-binding proteins (IGFPB) are proteins that form complexes with IGF. The formation of these complexes enhances or attenuates the function of IGF by prolonging the half-life of IGFs circulating in plasma and interfering with IGF binding to their receptors, reviewed in (Baxter and Twigg, 2009).

1.6.2. Insulin/IGF-I receptor substrates (IRS)

Unlike other RTK, insulin and IGF-I receptors do not interact directly with effectors, but in the majority of cases signal through insulin/IGF-I receptor substrates (IRS). The family of IRS is comprised of six members (IRS1-6) (Cai et al., 2003a; Fantin et al., 1998; Lavan et al., 1997; Sun et al., 1991; Sun et al., 1995). IRS1 and IRS2 have been studied extensively and were shown to be expressed by numerous cells and tissues, IRS 3-6 show limited tissue distribution and their role in signalling is not fully elucidated (Cai et al., 2003a). Phosphorylated tyrosines in activated IRS interact with (SH2) domain-containing enzymes and adaptor proteins such as the p85 regulatory unit of PI3K (Myers et al., 1992). IRS1 and 2 proteins have been implicated in various insulin/IGF-I-mediated physiological and pathological processes. The loss of IRS2 caused insulin resistance and diabetes, whereas the loss of IRS3 and 4 in mice had no effect on growth or metabolism (Fantin et al., 2000; Withers et al., 1998). The loss of IRS1 caused only mild insulin resistance in peripheral tissues, glucose intolerance and impaired growth (Araki et al., 1994; Tamemoto et al., 1994). Interestingly, in mice, liver-specific ablation using short hairpin RNAs against IRS1 enhanced the expression of genes involved in gluconeogenesis. Whereas, IRS2 ablation promoted the expression of genes involved in lipogenesis (Taniguchi et al., 2005), indicating that in the liver, at least, IRS1 and 2 have complementary, but not redundant roles in insulin/IGF-1 signalling. IRS activity, like that of insulin/IGF-I receptors, is inhibited by tyrosine phosphatases, such as SHP2 which binds to two phosphotyrosines on IRS1 C-terminus (Myers et al., 1998). Insulin elicits its biological effects through two major intracellular signalling pathways, the MAPK pathway (a.k.a. growth pathway) and the PI3K/Akt pathway, the so-called, metabolic pathway as shown in Figure 1.8. The MAPK/PKC pathway generally regulates insulin-mediated cell growth and mitogenesis.

The PI3K/Akt pathway mediates insulin metabolic effects. In the metabolic syndrome, the imbalance between these two pathways (suppression of PI3K/Akt and activation of MAPK and PKC) is considered the major cause of pro-inflammatory signalling up-regulation and reduced NO production, a hallmark of endothelial dysfunction (Kim et al., 2006). It was, however, postulated that insulin-activation of atypical isoforms of protein kinase C (aPKC) i.e. aPKCζ, acting downstream of PI3K are essential for the regulation of glucose transport in adipose and skeletal muscle tissues. Compromised activation of these enzymes has been reported to contribute to the development of insulin resistance (Standaert et al., 1997)

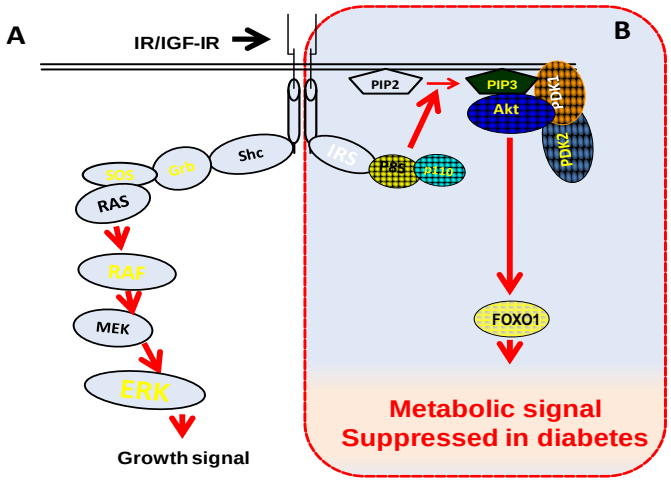


***Figure 1.8. Insulin signalling pathways**. The binding of insulin to the extracellular domain of the insulin receptor elicits a conformational change, which in turn leads to receptor autophosphorylation and tyrosine phosphorylation of intracellular protein substrates. Two main branching pathways are activated by insulin: **A**, The MAPK signalling cascade, in which Grb2/Sos leads to activation of Ras signalling, affecting, particularly cell proliferation. **B**, The IRS pathway, which leads to activation of kinases dependent upon the PI3K, such as Akt; Akt modulates enzyme activities that control glucose, lipid, and protein metabolism. PIP2, phosphoinositide 4,5 di-phosphate; PIP3, phosphoinositide 3,4,5 tri-phosphate; PDK1, phosphoinositide–dependent kinase–1; MEK, Mitogen-activated protein kinase kinase**, ERK, extracellular-signal-regulated kinase. FOXO1, forkhead boxO1.*

1.6.3. Phosphoinositide 3-kinase (PI3K) pathway.

Phosphatidylinositol 3-kinases (PI3K) are members of a conserved family of intracellular lipid kinases that phosphorylate the 3'hydroxyl group of membrane phosphatidylinositol (PtdIns). They have been shown to play a key role in diverse array of cellular processes, most notably cell proliferation, survival, growth, migration and metabolism induced by insulin/IGF-I and various other growth factors. PI3K are divided into three classes (I-III) according to their *in vitro* substrate specificity and sequence similarity. Class I PI3K have received the most attention, while relatively little is known about the other two classes. The class I PI3K are further divided into two subclasses IA and IB and exist as heterodimers. The class IA consist of a p110 (p110α, β, or δ) catalytic subunit and a p85 regulatory subunit (p85α, p55α, p50α, p85β, or p55γ), whereas, the class IB comprise the regulatory p101 and p100γ subunits. The regulatory subunits contain two SH2 domains that facilitate the high affinity interactions with tyrosine-phosphorylated pYMXM and pYXXM motifs in IRS proteins (Myers et al., 1992; Songyang et al., 1993; Yoakim et al., 1994). Class I PI3K are responsible for the production of phosphatidylinositol (3,4,5)-trisphosphate PtdIns(3,4,5)P_3, which is the major product resulting from growth factor–mediated activation of PI3K by RTKs (Cheatham et al., 1994; Nave et al., 1996) (Figure 1.9). (PtdIns(3,4,5)P_3) bind to pleckstrin homology (PH) domains of various signalling molecules, thus facilitating their recruitment to cellular membrane for activation (Lietzke et al., 2000). In the insulin/IGF-I metabolic pathway the phosphoinositide-dependent protein kinase 1 (PDK1) is a major downstream effector of PI3K and is essential for the activation of Akt/PKB (Alessi et al., 1997). PTEN, an endogenous phospholipid phosphatase, negatively regulates PI3K activity by inactivating PtdIns(3,4,5)P_3 through the dephosphorylation of the phosphoinositide ring on the

3'position (Maehama and Dixon, 1999) (Figure 1.9). The SH2-containing inositol 5' phosphatase-2 (SHIP2) acts in a similar manner as PTEN, but at the 5' position (Wada et al., 1999). Deletion of PTEN *in vivo* improves insulin sensitivity (Tang et al., 2005) and the loss of SHIP2 rescues mice from obesity-induced insulin resistance (Sleeman et al., 2005). The pharmacological inhibitors, Wortmannin (Ui et al., 1995) and LY294002 (Vlahos et al., 1994), or over-expression of PI3K mutants subunit or PTEN can all be used to block all metabolic actions of insulin.

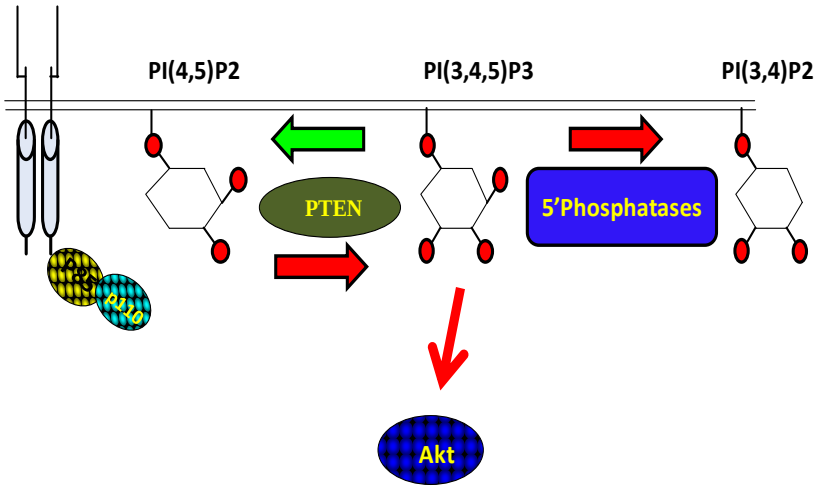


Figure 1.9. PTEN & 5'phophatases block the PI3K/Akt signalling pathway. *The synthesis of PtdIns(3,4,5)P_3 from PtdIns(4,5)P_2 via phosphorylation at the third position of the inositol ring by PI3K. PTEN blocks by catalysing the conversion of PtdInsP_3 back to PtdIns(4,5)P_2, whereas 5-phosphatases, such as the SHIP proteins, convert PtdInsP_3 to PtdIns(3,4)P_2. These two PI3K-dependent signals, PtdInsP_3 and PtdIns(3,4)P_2, share several downstream binding targets, including Akt.*

1.6.4. Protein kinase B PKB/Akt

Protein kinase B or Akt (PKB/Akt) is a serine/threonine protein kinase that is the major downstream target of the PI3K and plays a key role in multiple cellular processes, such as glucose metabolism (Zisman et al., 2000), cell survival (Cai et al., 2003b; Datta et al., 1997), and apoptosis (Cardone et al., 1998). Akt was originally identified as the oncogene in the transforming retrovirus, *vAKT8* (Bellacosa et al., 1991). In humans

there are three Akt genes that encode Akt-1/PKBα, Akt-2/PKBβ, and Akt-3/PKBγ. All three isozymes have similar structure and size, but are expressed differentially both at the mRNA and protein level (Altomare et al., 1998; Bellacosa et al., 1991; Okano et al., 2000). Akt-1 is the major isoform expressed in endothelial cells and is heavily implicated in the regulation of angiogenesis. Although deficiency of Akt-1 in mice inhibited physiological angiogenesis, it enhanced pathological angiogenesis (Chen et al., 2005; Somanath et al., 2006). The loss of Akt-1 function in the mouse leads to retarded growth, but does not impair metabolism (Cho et al., 2001b), whereas, Akt-2 knockout mice develop diabetes and exhibit insulin resistance due in part to inhibition of insulin-dependent gluconeogenesis (Cho et al., 2001a). Mutations in the Akt-2 kinase domain in humans cause severe insulin resistance and diabetes (George et al., 2004). Akt-3 is predominantly expressed in the brain and has no role in glucose metabolism (Tschopp et al., 2005).

The Akt family proteins contain a central kinase domain with specificity for serine/threonine residues in substrate proteins (Bellacosa et al., 1991). At the N-terminus of Akt there is the pleckstrin homology (PH) domain, which binds PtdIns(3,4,5)P_3 and mediates its recruitment lipids and other signalling molecules. This allows Akt translocation to cell membranes and its phosphorylation by phosphoinositide dependent kinase 1 (PDK1) at threonine 308 (Datta et al., 1996; Franke et al., 1997). Full activation of Akt requires a second phosphorylation on serine 473 by mammalian target of rapamycin complex 2 (mTORC2) (Sarbassov et al., 2005). The carboxyl terminus contains a hydrophobic and proline-rich domain (Datta et al., 1999). Upon activation by insulin or IGF-I, Akt mediates most of the metabolic actions of PI3K

through the phosphorylation of various effectors, including other signalling proteins and transcription factors such as glucose transporter 4 (GLUT4), NF- κB, forkhead transcription factors (Birnbaum, 1989; Brunet et al., 1999). In endothelial cells, there are a number of effectors that mediate cellular processes downstream of Akt, including protein synthesis via mTOR, and cell survival and metabolism though NF- κB and FOXOs (Manning and Cantley, 2007).

1.6.5. Forkhead transcription factors

1.6.5.1. Origin and structure

Forkhead transcription factors are a superfamily of proteins that play a major role in regulating the expression of genes involved in physiological processes such as the cell growth, proliferation, apoptosis, differentiation, longevity, embryonic development and stress resistance. The forkhead (fkh) gene was first discovered in Drosophila melanogaster, and there are now a large superfamily of >100 structurally-related genes (Weigel et al., 1989), which in humans consists of at least 143 members (Burgering, 2008). In recent years a consensus has been reached regarding the nomenclature leading to the classification of these proteins as forkhead box (Fox) transcription factors (Kaestner et al., 2000). For mouse, only the first letter should be capitalised i.e. FoxO1, for all other chordates the initial and subclass letters are in uppercase i.e FOXA3 (Kaestner et al., 2000). FoxO proteins were initially identified as tumour suppressor genes in certain cancers (Anderson et al., 1998; Davis et al., 1994; Galili et al., 1993). Due to sequence differences in the DNA binding domain, mammalian FoxO proteins are the most divergent subfamily of the FoxO proteins (Barthel et al., 2005; Kaestner et

al., 2000). These comprise FOXO1 (FKHR), FOXO3a (FKHRL1), FOXO4 (AFX) and FOXO6. FOXO1, FOXO3, and FOXO6 contain ~ 650 amino-acids, whereas the FOXO4 sequence is shorter ~ 500 amino-acids (Obsil and Obsilova, 2008). Despite the fact that these proteins are ubiquitously detected in various tissues, including the reproductive system, heart, lung, liver, skeletal muscle, pancreas, spleen, thymus and nervous system (Maiese et al., 2009), their distribution and function are tissue-dependent, suggesting specific functions for each FOXO in different cell types (Burgering, 2008). For example, FOXO1 is highly expressed in adipose tissue; FOXO4 is predominantly in skeletal muscle, FoxO3a in liver (Furuyama et al., 2000) and FOXO6 is selectively expressed in the brain (Hoekman et al., 2006).

As shown in Figure 1.10, FoxO proteins contain four domains a highly conserved forkhead DNA-Binding-Domain (DBD) located in the N-terminal region, a nuclear localisation signal (NLS) located just downstream of the DBD, a nuclear export sequence (NES) and a transactivation (TA) domain in C-terminal region. FOX proteins regulate transcription of target gene expression (suppression or activation) by binding to DNA through the structurally compact 100 amino-acid DBD, (a.k.a.forkhead domain; FKH). The hepatocyte nucleofactor 3γ (HNF3γ), contains a typical FKH consisting of three major α-helices, three beta sheets and two large, wing-like protein loops (Clark et al., 1993; Jin et al., 1998), thus conferring the name winged helix transcription factors. High affinity DNA binding studies have identified a consensus FOXO-recognition element (FRE) TT-(G/A)-T-T-(G/T)-(G/A)-(C/T) (Overdier et al., 1994; Pierrou et al., 1994). The optimal DNA-binding site for the FOXO proteins is 5'TTGTTTAC3'. More recently studies have shown FoxO recognition of more diverse DNA binding motifs (Gao et al., 2010).

1.6.5.2. Post-translational modifications of FoxO proteins

FOXO activity is tightly controlled by very complex signalling networks. FOXO function is regulated by their subcellular localisation, which can be modulated by phosphorylation, ubiquitylation and acetylation in response to different stimuli.

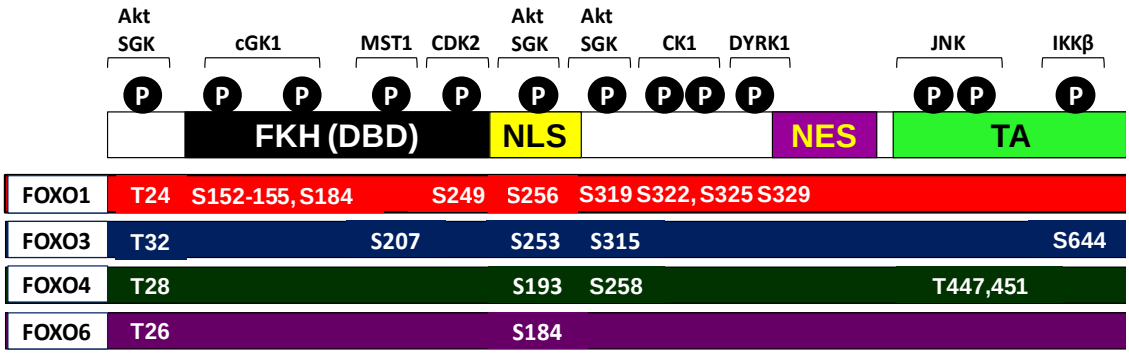


***Figure 1.10. FoxO transcription factor domains and phosphorylation sites**. The FoxO factors comprise a forkhead domain (FKH, the DNA binding domain), a nuclear localisation signal (NLS), a nuclear export sequence (NES) and a transactivation domain (TA). Modified residues after phosphorylation are indicated and amino–acid numbers are given for the individual isoforms. Adapted from (Van der Horst et al, 2007).*

1.6.5.2.1. Phosphorylation

A hallmark of the mammalian FOXO family is the presence of highly conserved sites for phosphorylation by Akt within and adjacent to their DBD (Biggs et al., 1999; Brunet et al., 1999; Kops et al., 1999). The PI3K/Akt signalling pathway is conserved between worms and mammals, and FOXO1, FOXO3, and FOXO4 are all targets of insulin, IGF-I and growth factor mediated PI3K/Akt phosphorylations. For FoxO1 these are at Thr 24, Ser 256, and Ser 319. FoxO phosphorylation by PI3/Akt can be blocked by LY294002, and expression of dominant-negative mutant forms of PI3K and Akt (Biggs et al., 1999; Brunet et al., 1999). In response to growth factors, the PI3K/Akt pathway inhibits FOXO-dependent transcription through the phosphorylation and subsequent

sequestration of FOXO factors in the cytoplasm (Figure 1.11) (Biggs et al., 1999; Brunet et al., 1999). Mutation of Akt phosphorylation sites on FOXO or inhibition of PI3K/Akt leads to stable nuclear localisation of FOXO (Biggs et al., 1999; Brunet et al., 1999).

FOXO proteins can also be phosphorylated by the serum and glucocorticoid-induced kinase 1 (SGK1) (Brunet et al., 2001), which translocates to the nucleus upon activation by PI3K in a similar fashion to Akt. Although SGK and Akt phosphorylate common sites on FOXO proteins, Akt phosphorylates Ser 256 on FoxO3a, whereas SGK1 favours Ser 319. SGK-1 was shown to mediate glucocorticoid cellular cell survival through the inhibition of FoxO3a activity (Mikosz et al., 2001; Wu et al., 2006), and SGK3 also directly phosphorylates and inactivates FoxO1 (Liu et al., 2000)

Unlike PI3K/Akt, the c-Jun-N-terminal-Kinase (JNK) phosphorylates FOXOs on alternative residues to induce their activation and promote their nuclear localisation, in response to oxidative stress, even in the presence of growth factors (Essers et al., 2004). Treatment of cells with hydrogen peroxide (H_2O_2) triggers the activation of JNK pathway leading to the phosphorylation of Thr 447 and Thr 451, activation and nuclear retention of FoxO4 resulting in the induction of anti-oxidative genes such as manganese superoxide dismutase (MnSOD) (Essers et al., 2004).

Other pathways that have been implicated in the phosphorylation of FoxO proteins are the oxidative-stress-regulated mammalian sterile 20-like (MST1), and another kinase and JNK activator, which was also shown to bind and phosphorylate FoxO3a on serine

207 directly, or through JNK both *in vitro* and *in vivo* (Graves et al., 1998; Henderson and Johnson, 2001). The cyclin-dependent kinase-2 (CDK2) phosphorylates of FOXO1 on Ser 249 and 298 leading to its exclusion from the nucleus, hence, inhibition of its activity. This was considered as an apoptotic response to DNA damage that can be blocked by oxidative stress, which was shown to inhibit CDK2 activity (Huang et al., 2006). In addition, casein kinase 1 (CK1), which is a serine/threonine kinase has also been shown to phosphorylate FoxO proteins. This effect was shown to be Akt-dependent (Seol and Kim, 2003). More recently, the retention of FOXO3a in the nucleus, in some cancers, led to the discovery that IκB Kinase (IKK) phosphorylates FOXO3a at serine 644 in an Akt-independent fashion (Hu et al., 2004). Furthermore, the dual-specifictiy tyrosine–phosphorylated and regulated kinase (DYRK), which plays a role in the neural proliferation in central nervous development also phosphorylates FoxO1 on serine 329 *in vitro* thereby promoting its nuclear exclusion and inhibiting its transcription activity (Woods et al., 2001)

1.6.5.2.2. Acetylation

Oxidative stress increases FoxO acetylation by the CREB-binding protein (CBP) and p300 (Brunet et al., 2004; Frescas et al., 2005). FoxO1 interaction with proteins with intrinsic histone acetyl-transferase (HAT) activity such as the co-activators p300 and CBP enhanced their activity (Perrot and Rechler, 2005), while disruption of these interactions with mutant CBP lacking acetyl-transferase activity inhibits FOXO1 activity (Daitoku et al., 2004; Nasrin et al., 2000). Furthermore, the NAD-dependent histone deacetylase SIRT1 (silent mating type information regulation 2 homologue 1), deacetylates FoxO proteins in the nucleus under stress conditions to induce the

transcription of anti-oxidative genes (Brunet et al., 2004; Kitamura et al., 2005). The expression of SIRT1 shifts the balance in FoxO function from apoptosis to survival (Brunet et al., 2004; Motta et al., 2004).

1.6.5.2.3. Ubiquitylation

The proteasome are protein complexes that exist in the cytoplasm and nucleus of eukaryotic cells. The function of these proteins is the degradation of unneeded or misfolded proteins by proteolysis. The process of degradation by ubiquitin proteins involves the binding of multiple ubiquitin molecules to the target protein; and degradation of the tagged protein by the 26S proteasome or lysosomes (Ciechanover, 1998). FoxO proteins expulsion from the nucleus to the cytoplasm subjects them to ubiquitination-dependent proteosomal degradation. FoxO1 and FoxO3 degradation by ubiquitylation and subsequent degradation by proteosomes is promoted by activated Akt (Plas and Thompson, 2003). The use of lactacystin, a proteosome inhibitor, prevents PDGF stimulated inactivation of FoxO1 and exclusion from the nucleus in fibroblasts. The PI3K inhibitor, LY294002, has similar effects indicating that proteosome-mediated degradation of FoxO1 is Akt-dependent as FoxO1 degradation requires the phosphorylation on Ser 256 by Akt (Aoki et al., 2004). Interestingly, FoxO3a poly-ubiquitination through murine double minute (MDM2) oncogene is promoted by FoxO3a phosphorylation by ERK.

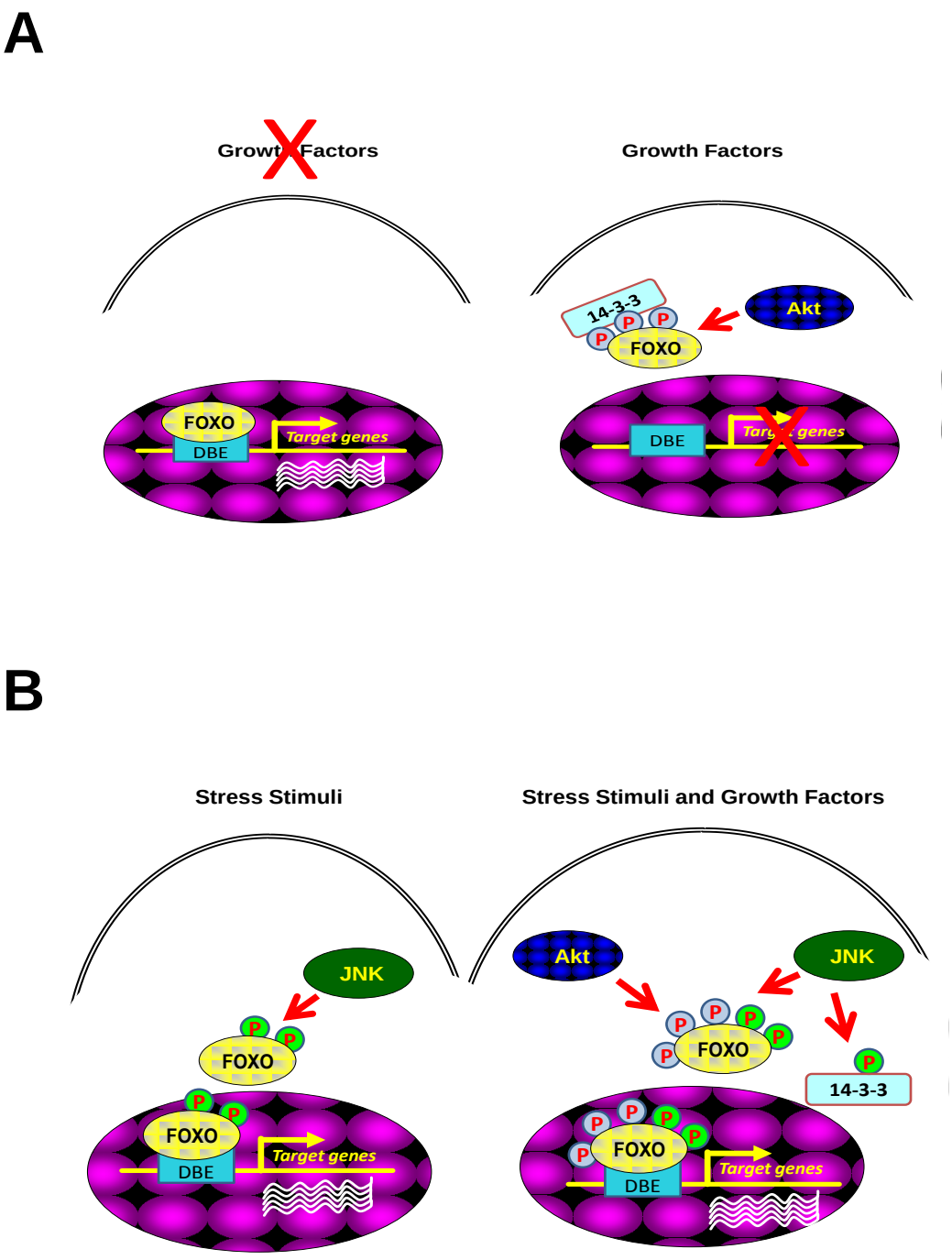


Figure 1.11. FOXO regulation by growth factors and stress stimuli. ***A****, In response to growth factors, the PI3K/Akt pathway inhibits FOXO-dependent transcription through the phosphorylation and subsequent sequestration of FOXO factors in the cytoplasm. B, Stress stimuli are sufficient to overcome the cytoplasmic sequestration of FOXO factors. In response to stress stimuli, JNK phosphorylates FOXO factors, which causes the nuclear translocation of FOXO proteins. Although FOXO phosphorylation by JNK does not directly inhibit the binding of 14-3-3 proteins, JNK can phosphorylate 14-3-3 directly, thus releasing 14-3-3 substrates. Adapted from El Greer et al, 2005*

1.7. FoxO in the regulation of angiogenesis and endothelial cell biology.

The role of FoxO factors in the regulation of angiogenesis and endothelial cell function has been determined in mice using gene loss or gain of function approaches in the whole genome or strictly in the endothelial lineage. FoxO1-deficient mice die on embryonic day 10.5 as a result of poorly developed blood vessels, pointing to the indispensible role that FoxO1 plays in the formation of functional vascular system (Furuyama et al., 2004; Hosaka et al., 2004). Conversely, FoxO3a$^{-/-}$ mice are viable and exhibit abnormal ovarian follicular development leading to age-dependent reduced infertility (Castrillon et al., 2003). Whilst, FoxO4$^{-/-}$ mice show no obvious phenotype (Castrillon et al., 2003; Hosaka et al., 2004). Conditional combined FoxO1, FoxO3a and FoxO4 knockout mice exhibit excessive endothelial cell proliferation, tissue-selective haemangioma and premature death (Paik et al., 2007), suggesting that these transcription factors function as angiogenesis inhibitors and tumour suppressors. In another study (Potente et al., 2005), the deletion of FoxO3a enhanced angiogenesis and rescued hind limb ischemia by abrogating the suppressive effects of FoxO3a on eNOS expression. Both FoxO1 and FoxO3a, but not FoxO4, bind directly to the eNOS promoter and siRNA-mediated silencing of FoxO1 and FoxO3a enhanced VEGF-mediated tube formation on growth factor-reduced Matrigel and migration of endothelial cells (Potente et al., 2005). In line with these observations, over-expression of mutant FoxO1 and FoxO3a inhibited blood vessel formation and endothelial cell migration (Potente et al., 2005). Interestingly, angiopoietin-1 (Ang-1), another endothelial cell survival factor and a regulator of vascular maturation and stability, inactivates FoxO1 via its receptor Tie2 and downstream activation of Akt. Constitutively-active FoxO1 induces expression of the Ang-1 antagonist, Ang-2, which blocks the activation of PI3K/Akt leading to blood vessel destabilisation and

remodelling (Daly et al., 2006). This positive feedback loop is another way through which FoxO regulates its own activity and tightly modulates expression of genes involved in angiogenesis. Taken together, FoxO factors and in particular FoxO1, are major regulators of angiogenesis.

1.8. FoxO regulation of metabolism

Insulin acts as the primary regulator of the glucose and energy homeostasis by stimulating the uptake of glucose and its subsequent metabolism (glycolysis and glycogen synthesis). FOXO1 was identified as the major transcription factor mediating metabolic-regulatory effects of insulin (Accili and Arden, 2004). FOXO1 is the most abundant isoform expressed in insulin–responsive tissues and negatively regulates insulin insensitivity in liver, adipose tissue and in pancreas by mediating insulin-induced changes in gluconeogenic enzymes (Barthel et al., 2005; Nakae et al., 2001).

1.8.1. FoxO1 in the liver

When energy in abundant, the liver is the major organ for glucose up-take and storage. When energy levels are low, glucose synthesis is induced by the up-regulation of gluconeogenic enzymes, glucose-6-phosphatase (G6Pase) (Nakae et al., 2001; Schmoll et al., 2000) and phosphoenolpyruvate carboxykinase (PEPCK) (Yeagley et al., 2001). FOXO1 promotes gluconeogenesis. Over-expression of constitutively-active FoxO1 in the liver results in the up-regulation of gluconeogenesis genes (G6Pase, PEPCK) (Zhang et al., 2006), whereas liver-specific FoxO1 knockout, or injection of FoxO1

antisense oligonucleotides in the liver and adipose tissue improved insulin sensitivity and glucose tolerance in both lean and diet-induced obese mice.

FOXO1 also modulates triglyceride metabolism through the up-regulation of apoliproprotein CIII (apoCIII). ApoCIII is a very low density aliproprotein predominantly found in the liver and its main function is as an endogenous inhibitor of lipoprotein lipase (LPL) mediated hydrolysis of lipids into free fatty acids and monoacylglycerol. Transgenic mice over-expressing apoCIII develop hypertriglyceridemia, one of the main causes of atherosclerosis, due to the blockade of triglyceride-rich particles uptake in the liver, which promotes diet-induced obesity and insulin resistance (Duivenvoorden et al., 2005; Ito et al., 1990). Mice transduced with constitutively-active FoxO1 adenovirus exhibit increased levels of apoCIII and develop hypertriglyceridemia (Altomonte et al., 2004). Taken together, these data show that FoxO1 is focal point at which several pathways regulating metabolism converge in the liver.

1.8.2. FoxO1 in adipose tissue

Obesity-induced insulin resistance is a major contributor to the development of diabetes mellitus and atherosclerosis (Saltiel and Kahn, 2001). FoxO1 is highly expressed in both white and brown adipose tissue and is a major target for insulin-mediated regulation of metabolism in these tissues (Nakae et al., 2003). FoxO1 plays an essential role in adipocyte differentiation and the knockout of any of the insulin signalling pathways resulted in impaired FoxO1-mediated pre-adipocyte differentiation (Accili

and Taylor, 1991; Miki et al., 2001; Tseng et al., 2004; Xu and Liao, 2004; Yun et al., 2008). Constitutively-active FoxO1 over-expression blocks this differentiation, while, dominant-negative FoxO1 restored pre-adipocyte differentiation in IR knockout mice (Nakae et al., 2003). FoxO1 control of pre-adipocyte differentiation is mediated by the cell cycle inhibitor p21 and peroxisome proliferator-activated receptor (PPARγ), a nuclear receptor protein that acts as a transcription factor (Dowell et al., 2003), FoxO1 binding to PPARγ decreases insulin sensitivity (Armoni et al., 2006; Armoni et al., 2003). SIRT2, the predominant sirtuin family member expressed in adipocytes, activates FoxO1 enhancing its phosphorylation and nuclear exclusion, thus promoting adipocyte differentiation (Jing et al., 2007).

1.8.3. FoxO1 in skeletal muscle

FoxO1 inactivation enhances skeletal muscle growth and differentiation through the increase in fusion of monucleated myotubes into myoblasts (Bois and Grosveld, 2003; Hribal et al., 2003; Tureckova et al., 2001). Disruption of IGF-I/PI3K/Akt signalling pathway in muscle inhibits protein synthesis and causes atrophy (Bodine et al., 2001). Mice expressing constitutively-active FoxO1 in skeletal muscle are underweight, with smaller skeletal muscles (Kamei et al., 2004).

1.8.4. FoxO1 in pancreatic β-cells

β-cell development, proliferation and function are regulated by the insulin/IGF-I/PI3K/Akt signalling pathway through FoxO1, the most abundant forkhead transcription factor expressed in pancreatic β-cells (Kitamura et al., 2002). In $IRS2^{-/-}$ transgenic mice, failure of β-cells leads to increased glucose levels combined with peripheral insulin resistance and diabetes (Kubota et al., 2000; Withers et al., 1998), which can be rescued by the deletion of a FoxO1 allele (Kitamura et al., 2002). FOXO1 also regulates β-cell proliferation through suppression of PFDX-1 transcription which mediates β-cell survival and insulin-coding gene, Ins2 expression in response to oxidative stress in insulin resistance (Kitamura et al., 2002; Kitamura et al., 2005).

1.9. The metabolic syndrome (MBS)

Metabolic syndrome, a.k.a syndrome X, is a disorder that encompasses diabetes, glucose intolerance, obesity, dyslipidemia and hypertension (Reaven, 2005). Individuals with metabolic syndrome are more susceptible to developing cardiovascular disease (CVD) and type 2 diabetes (Dekker et al., 2005; Lorenzo et al., 2003). Insulin resistance in liver and adipose tissues is considered the chief underlying cause of most of the aforementioned disorders (Grundy et al., 2004). In metabolic tissues, the loss of insulin responsiveness results in continuous secretion of insulin by pancreatic β-cells causing hyperinsulinemia, combined with failure to suppress glucose levels over time, this leads to hyperglycaemia and overt type 2 diabetes (Petersen and Shulman, 2006).

Several mechanisms have been postulated for insulin resistance, including inadequate insulin secretion, mutation of the insulin receptor or one of the IRS proteins, but the general consensus is that defective insulin signalling downstream of insulin receptor in insulin-sensitive tissues is the main cause. The suppression of the PI3K/Akt signalling pathway has been shown in the skeletal muscle of obese patients (Bjornholm et al., 1997; Goodyear et al., 1995), skeletal muscle and liver tissues of obese and hyperglycaemic *ob/ob* mice (Folli et al., 1993; Kerouz et al., 1997). The ablation of the hepatic insulin receptor causes severe hyperglycaemia and glucose intolerance in mice (Michael et al., 2000). In cardiovascular disorders, such as hypertension, coronary artery disease and atherosclerosis, insulin resistance is a common finding (Ford, 2005). In addition to its essential role in glucose and lipid metabolism, insulin has other important vascular functions, the impairment of which causes endothelial dysfunction, a prominent component of all the aforementioned cardiovascular disorders.

1.10. Endothelial dysfunction

Endothelial dysfunction is a key factor in the pathogenesis of vascular disease in metabolic disorders i.e. diabetes mellitus and obesity (De Caterina, 2000). As shown in Figure 1.12, under normal physiological conditions, in endothelial cells, insulin activation of the PI3K/Akt pathway induces NO production, which promotes vasodilatation, this leads to increased blood flow and glucose uptake by skeletal muscle (Baron and Clark, 1997). In hyperglycaemia, impaired insulin signalling leads to reduction in NO bioavailability, disruption of blood flow, and increase in vascular permeability, resulting in the loss of endothelial integrity and function. The activation of the PKC pathway by insulin leads to the over-expression of adhesion and pro-

inflammatory molecules such as ICAM-1, VCAM-1, E-selectin and vasoconstrictor endothelin 1 (ET-1) (Montagnani et al., 2002; Potenza et al., 2005). This shift tips the endothelium into a vasoconstrictor, pro-thombotic and pro-inflammatory state (Potenza et al., 2009b).

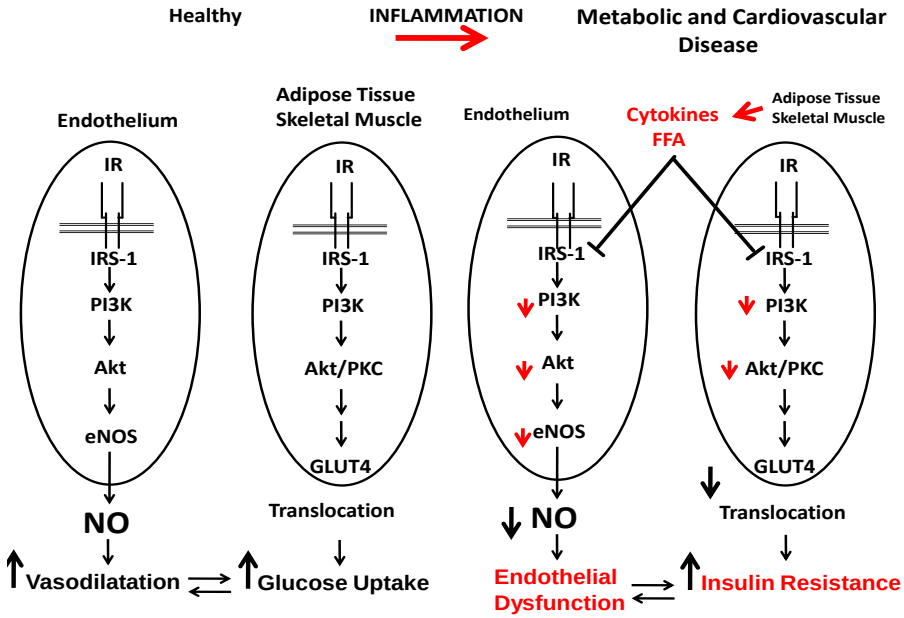


Figure 1.12. Mechanisms of endothelial dysfunction in vascular tissues and insulin resistance in metabolic tissues. *PI3K-dependent insulin signalling pathways in metabolic and vascular tissues synergistically marry metabolic and vascular physiology under healthy conditions and contribute to synergistic coupling of inulin resistance and endothelial dysfunction (Adapted from Kim et al, 2006).*

1.11. Mechanisms of hyperglycaemia-induced endothelial dysfunction

The association between hyperglycaemia and endothelial dysfunction in diabetic vascular complications such as diabetic retinopathy, nephropathy and atherosclerosis is well established (De Caterina, 2000). Hyperglycaemia-induced endothelial dysfunction is thought to be a consequence of oxidative stress due to an increased production of reactive oxygen species in the mitochondria. As shown in Figure 1.13, there are 4 pathways by which hyperglycaemia induces oxidative stress.

1.11.1. The polyol/sorbitol/aldose reductase pathway

In most cells under normoglycaemic conditions, non-metabolised excess glucose enters the polyol pathway where aldose reductase reduces it to sorbitol. This is later metabolised to fructose by sorbitol dehydrogenase. This reaction also oxidises NADPH to NADP+ and reduces NAD+ to NADH. NADP+ and NAD+ are essential cofactors for NO synthesis (Dagher et al., 2004). In the hyperglycaemic state, increased glucose levels combined with the increased affinity of aldose reductase for glucose result in sorbitol accumulation and decreased NADP+ and NAD+ available for NO production, culminating in increased oxidative stress and cell death (Gabbay, 1975). This pathway has been implicated in diabetic retinopathy in humans and rats (Dagher et al., 2004).

1.11.2. Increased hexosamine pathway activity

Hyperglycaemia-induced activation of the hexosamine pathway has been shown to contribute to the complications of diabetes. In this pathway, fructose 6-phosphate is diverted from the glycolysis pathway into a signalling pathway where it is converted into UDP-*N*-acetylglucosamine by glutamine:fructose 6-phosphate amidotransfeRase

(NGFAT). The addition of UDP-*N*-acetylglucosamine onto serine and threonine residues results in altered enzyme function i.e. the inhibition of Akt phosphorylation in human endothelial cells causes reduction in insulin-mediated NO production, thus endothelial dysfunction (Federici et al., 2002).

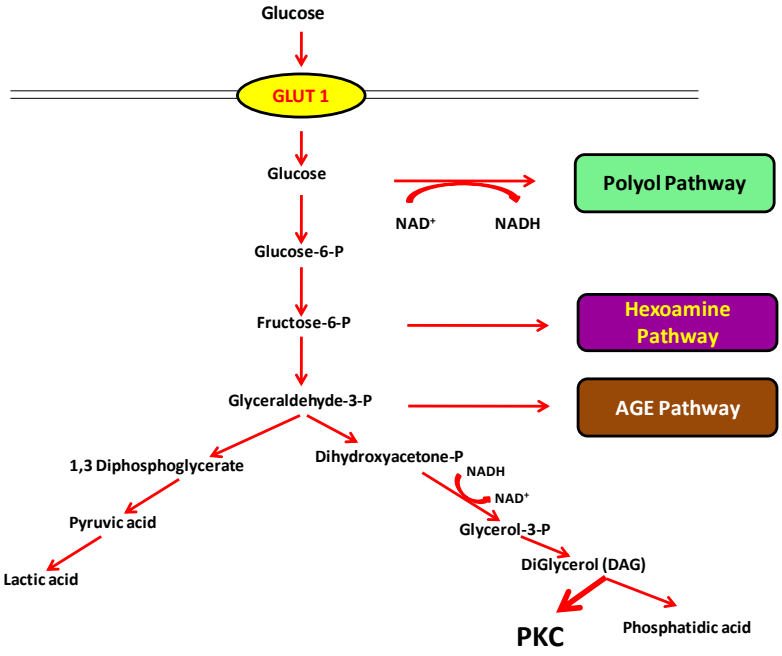


Figure 1.13. Mechanisms of hyperglycaemia-induced endothelial dysfunction. *Excessive glucose enters endothelial cells via GLUT1 in an insulin-independent manner, this lead to activation of the polyol pathway, hexoamine pathway, AGE pathway, and PKC pathway. Glucose transporter 1 (GLUT1). Advanced glycation products (AGE).*

1.11.3. Increased intracellular production of advanced glycation end products (AGE).

In diabetes, hyperglycaemia is the main trigger of the production of advanced glycation end products (AGE) through non-enzymatic reactions between glucose, or its derivatives, with proteins (Stevens et al., 1977). Over-production of these molecules can cause cell damage and induce inflammation though their ability to alter the function of intracellular (i.e. transcription factors), extracellular (i.e. receptors and integrins) or blood-born proteins (i.e. albumin) (Giardino et al., 1994; Li et al., 1996; McLellan et al., 1994). Increased accumulation of AGE has been shown in the vitreous of the eye of diabetic patients (Stitt et al., 1998), and in the retinal vasculature of diabetic and AGE-infused rats (Stitt et al., 1997).

1.11.4. The DAG/PKC pathway

Induction of *de novo* diacylglycerol (DAG) synthesis by [chronic] hyperglycaemia, which positively regulates PKC activity, has been associated with vascular complications of diabetes. DAG up-regulation and PKC activation has been shown in tissues from heart, aorta, retina from diabetic patients and animal models of diabetes, and in cultured endothelial cells exposed to hyperglycaemia (Inoguchi et al., 1992; Shiba et al., 1993). Endothelial dysfunction in vascular injuries is believed to result through the disruption of blood flow by decreasing NO activity and up-regulation of the expression of pro-inflammatory factors, such as ET-1 and adhesion molecules ICAM-1, VCAM-1, E-selectin and various pro-inflammatory cytokines.

1.12. Hyperglycaemia and oxidative stress

Hyperglycaemia-induced over-production of superoxide by mitochondrial electron transport chain has been recognised as the unifying mechanism that acts upstream of all the aforementioned pathways (Figure 1.14) (Nishikawa et al., 2000). Diabetic rats have increased levels of superoxide (Sartoretto et al., 2007) a highly reactive molecule that contributes to oxidative stress generation. Oxidative stress is a prominent feature of endothelial dysfunction. Inhibition of hyperglycaemia-induced ROS blocked the activation of polyol, hexosamine and PKC pathways and AGE formation (Guzik et al., 2002). In endothelial cells ROS induce cell apoptosis, senescence, activation of the pro-atherogenic inflammatory pathways and abrogation of NO bioavailability (Madamanchi et al., 2005), all key features of endothelial dysfunction.

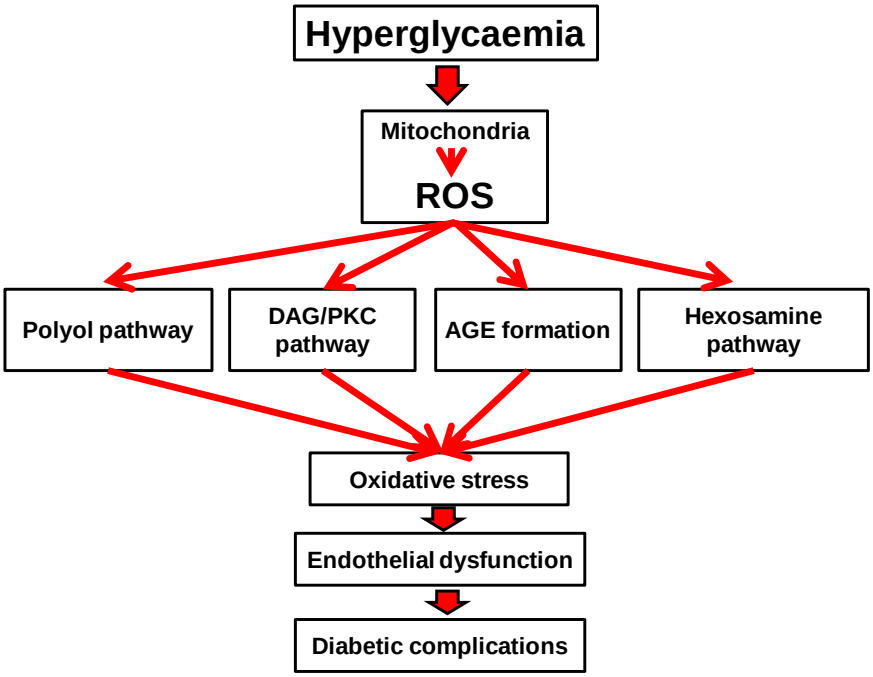


Figure 1.14. Mechanisms of hyperglycaemia–induced endothelial dysfunction and in the end diabetic complications. *Adapted from (Van den Oever et al., 2010)*

1.13. Mouse models of diabetes and obesity

Several mouse models of diabetes and obesity have been established. The tubby mouse model develops late onset obesity with insulin resistance, but how it is developed, the mechanism for it is still unknown (Kleyn et al., 1996). In the carboxypeptidase E mouse model, the disruption of this enzyme's function blunts the melanocortin responsiveness to leptin leading to the development of hyperglycaemia and the onset of obesity (Naggert et al., 1995). Another model of animal model of diabetes is the C57BL/KsJ mice (*db/db*) mouse which is characterised by autosomal recessive leptin receptor deficiency (Leibel et al., 1997). This mouse has metabolic abnormalities that culminate in systemic hyperglycaemia, insulin resistance and phenotypic obesity

(Garris, 1989; Garris and Garris, 2003). The leptin-deficient *ob/ob* mouse has been used extensively as a model for obesity, insulin resistance, lipotoxicty and type-2 diabetes (Lindstrom, 2007). The *ob/ob* phenotype was identified in an out bred colony of mice in 1949 (Ingalls et al., 1950), and backcrossed onto the C57Bl/6 background. *Ob/ob* mice have a mutation in the leptin gene that was identified by positional cloning, (Zhang et al., 1994) and prevents its expression. The mice are highly leptin sensitive and the *ob/ob* phenotype can be completely reversed through transfer of the leptin gene, or administration of leptin (Pelleymounter et al., 1995). Initially *ob/ob* mice are indistinguishable from their lean littermates, but within two weeks they grow rapidly and start to develop severe obesity through uncontrolled food intake leading to excessive adipose accumulation, hyperlipidaemia, steatosis, severe hyperglycaemia (despite hyperinsulinaemia) and inflammation. They show a dramatic increase in the numbers of insulin producing β-cells driven by the increased demand for insulin. *Ob/ob* β-cells produce large quantities of insulin, as they lack the inhibitory effect of leptin, which leads to hyperinsulinaemia. However, insulin production is thought to be insufficient as the mice start to develop hyperglycaemia at four weeks of age which peaks at between 3-6 months before declining in old age (Westman, 1968). The severe insulin resistance which results in reduced PI3 kinase activity particularly in muscle (Folli et al., 1993) and leads to elevated FOXO1 activity (Behl et al., 2009).

1.14. Rationale

Evidence from both patients and animal studies clearly supports the role of PlGF and VEGFR-1 in the promotion of pathologies including atherosclerosis, diabetic retinopathy, arthritis, metabolic syndrome and cancer. Although considerable work has been undertaken to understand to role of PlGF in pathology, little is known about the mechanisms underpinning the regulation of its expression in these disorders. Unravelling these mechanisms/pathways will increase our knowledge of the biology of the ligand and its receptor, and may be beneficial for developing novel therapies. The overall aim of my studies was to identify the mechanisms involved in the up-regulation of PlGF and VEGFR-1 expression both in growth factor-driven angiogenesis and in pathologies.

The specific objectives of this thesis were to:

- Determine the effect of insulin-like growth factor-I and hyperglycaemia on PlGF expression in endothelial cells.

- Delineate signalling pathways, in particular PI3K/Akt, important for the regulation of PlGF expression under these conditions.

- Identify transcription factors regulating PlGF and VEGFR-1 gene expression.

- Determine the effect of hyperglycaemia in a mouse model of insulin resistance, on PlGF expression.

Investigations *in vitro* were performed on primary cultures of HUVEC isolated in-house and human aortic endothelial cells (HAEC) from commercial sources. These are normal human endothelial cells that can be passaged four times without losing expression of endothelial cell markers (Jaffe et al., 1973). HUVEC have been used in many *in vitro* studies examining cell signalling in the endothelium and regulation of VEGFR function and expression. Bovine aortic endothelial cells (BAEC) are primary endothelial cells that can be cultured more readily and passaged many times before losing their phenotype and were prepared as described in Sattar et al., (1994).

To examine the link between PI3K/Akt activity and PlGF expression, pharmacological inhibitors such as LY294002, complementary methods were also employed, as inhibitors are rarely complete specificity. Recombinant, replication defective adenoviruses were used to transduce HUVEC with dominant-negative or constitutively-active forms of Akt, PTEN and FOXO1. With this method of gene transfer up to 100% efficiency can be obtained in non-dividing cell populations. Akt activation was also achieved with IGF-I, which mimics insulin activities *in vitro.* siRNA gene silencing is a very specific, efficient and easy way to block protein synthesis and has been optimised in our laboratory for use in HUVEC (Al-Ani et al., 2010). siRNA was used to silence Akt-1, the predominant Akt isozyme expressed in endothelial cells. To examine the effect of hyperglycaemia on PlGF expression *in vivo, ob/ob* mice at 16-19 weeks of age, which are severely hyperglycaemic and insulin-resistant, were used (Garthwaite et al., 1980; Mayer et al., 1953). These are reported to show reduced PI3K /Akt activity (Folli et al., 1993; Kerouz et al., 1997).

The apparent involvement of PI3K/Akt and the overwhelming evidence implicating FOXO1 in the regulation of metabolism (Accili and Arden, 2004) provided a rationale to test the role of FOXO1 in PlGF expression in HUVEC. siRNA to silence FOXO1 was used in studies examining the effects of hyperglycaemia *in vitro* (Tanaka et al., 2009). *In vivo* studies to examine FOXO1 regulation of PlGF expression were performed using FVB/N mice, injected with adenovirus expressing constitutively-active FOXO1 (Nakae et al., 2003). In addition to assessing circulating levels of PlGF in plasma, the livers of these mice were examined by qPCR and ELISA as the majority of systematically delivered adenovirus is filtered out by the liver. To examine whether FOXO1 binds directly to PlGF and/or VEGFR-1 genes, chromatin immunoprecipitation (ChIP) assays have been employed. PCR amplification of immunoprecipitated DNA fragments bound to the relevant transcription factor can provide direct evidence of the transcription factor binding to the promoter of gene of interest. VEGFR-1 promoter constructs encoding the identified FOXO1 binding site were used to demonstrate the ability of FOXO1 to activate VEGFR-1 expression. HUVEC are difficult to transfect with plasmids, therefore, BAEC were used with plasmid constructs expressing constitutively-active FOXO1, and FOXO3a to test the selectiveness of FOXO1 in the regulation of VEGFR-1 expression in endothelial cells

The up-regulation of PlGF expression following VEGF stimulation in endothelial cells was demonstrated previously (Yao et al., 2005; Zhao et al., 2004), but the pathways and mechanism(s) regulating this process have not been fully elucidated. The roles of endogenous (autocrine) and exogenous (paracrine) VEGF and the naturally occurring PlGF:VEGF heterodimer in the regulation of PlGF release have been explored, using

siRNA-mediated knockdown and recombinant adenoviruses over-expressing mouse $VEGF_{164}$ and PlGF-2.

Chapter 2

Materials & Methods

2.1. Materials

The suppliers of all purchased chemicals, reagents used are detailed in Appendix I. Details of antibodies and suppliers are in Appendix II. Equipment and suppliers are listed in Appendix III. Protocols for preparation of solutions and buffers are present in Appendix IV.

2.2. Methods

2.2. Tissue and cell culture

All cell and tissue culture medium and supplements were purchased sterile or filtered through a 0.22 μm filter. Distilled water and calcium and magnesium [Ca^{2+} and Mg^{2+}] free Dulbecco's phosphate buffered saline (PBS – formula in APPENDIX IV) were autoclaved before use. Tissue culture-treated plastics were purchased sterile. Glassware was washed and autoclaved prior to use. Cell culture medium was stored at 4^{o}C and used within 3 months.

2.2.1. General maintenance of primary cells and cell lines

Routine maintenance of cell cultures was carried out in sterile class II cabinet. Cell cultures were maintained in humidified incubators in an atmosphere of 95% air, 5% CO_2 at 37^{o}C. Cells were grown in 75 cm^2 tissue culture flasks containing relevant medium supplemented with 5%, 10%, or 20% foetal bovine serum (FBS) and 2 mM L-

glutamine, 10 U/ml Penicillin and 0.1 µg/ml Streptomycin (GPS). The medium was changed every 2-3 days and on reaching confluence, cells were subcultured by aspiration of the medium from the cell monolayer followed by two washes with 10 ml of PBS to remove FBS. Cells were detached by 2-5 minute incubation with 1 ml of 0.5 g/l porcine trypsin and 0.2 g/l ethylenediaminetetraacetic acid (EDTA) in Hanks′ balanced salt solution with phenol red (HBSS) at 37^{o}C. Once detached, 10 ml of growth medium was added to inactivate trypsin. The cell suspension was transferred to a sterile 15 ml conical tube and centrifuged at 100 x g for 5 minutes to pellet the cells. Cell pellet was resuspended in 10 ml of growth medium. Cells were then either split between new culture flasks, as required to maintain stocks, or counted using haemocytometer in preparation for experiments requiring a defined number of cells per well (cpw) prior to seeding in 25 cm^2 flasks or 6, 12 or 24 well tissue culture plates. Details of cell seeding densities for human umbilical vein endothelial cells (HUVEC), the most commonly used cell type in this study, are shown in Table 2.1. Each time cells were subcultured they were assigned an ascending passage number.

Plates/ flasks	**Surface area (mm^2)**	**Cell number**	**Approximate cell number at confluency**	**Trypsin/EDTA vol (ml)**	**Volume of medium (ml)**
T-25	2500	250 x 10^3	1 x 10^6	0.5	3-5
T-75	7500	1 x 10^6	2.5 x 10^6	1	10-15
T-150	15000	2 x 10^6	5 x 10^6	2	15-30
6-well	962	0.32 x 10^6	1 x 10^6	0.3	1-3
12-well	401	0.16 x 10^6	0.25 x 10^6	0.2	1-2
24-well	200	0.08 x 10^6	0.12 x 10^6	0.1	0.5-1
96-well	50	0.02 x 10^6	0.05 x 10^6	0.05	0.1-0.2

Table 2.1. Plating density of HUVEC used for experimental procedures.

2.2.2. Cell cryopreservation

Reserve stocks of each cell type used was maintained through cryopreservation**.** Confluent monolayer of cells to be preserved were trypsinised and cell pellet resuspended at $1x10^6$ cells/ml in pre-cooled culture medium containing GPS, 45% (v/v) FBS and 10% (v/v) dimethyl sulphoxide (DMSO). Cell suspension was transferred to sterile cryovials labelled with cell type, passage number, and the date of storage. Freezing of cells was carried out at a rate of 1^oC/minute using 'MrFrosty' containing isopropyl alcohol placed in a -80^oC freezer prior to long-term storage in liquid nitrogen at -196^oC. To retrieve the cryopreserved cells, cryovials were thawed immediately in a water bath at 37^oC, transferred to a 15 ml tube and centrifuged for 5 mins at 100 x g and the pellet resuspended in complete growth medium and transferred to tissue culture flasks containing the appropriate growth medium. Cells were incubated for 16 hours to allow cell attachment followed by a wash with PBS to remove all traces of DMSO and fresh growth medium was added.

2.3. Details of primary cells and cell lines used

2.3.1. Human umbilical vein endothelial cell isolation and culture

Human umbilical vein endothelial cells culture Informed consent was obtained from healthy pregnant women undergoing elective caesarean section at Birmingham women's hospital under ethical approval obtained by Dr Peter Hewett [RCS10-0546.APP/N10-014]. Cords were collected on the day of surgery and washed with PBS to remove excessive blood and a 15-20 cm long section with no obvious clamp trauma

selected. The umbilical vein was identified, cannulated with a glass cannula at one end prior to flushing the vein with PBS to remove any blood clots. The cord was then perfused with 20 ml of type V collagenase (1 mg/ml) in HBSS and clamped at both ends. After a 20 minute incubation at 37^{o}C, detached endothelial cells were collected by flushing the collagenase solution through the umbilical vein with HUVEC complete growth medium 199 (M199) containing GPS, 20 ng/ml epidermal growth factor (EGF), 2.5 ng/ml FGF-2 and 20% FBS. The cell suspension was then centrifuged at 100 x g for 5-10 minutes and the resulting cell pellet resuspended in 12 ml of HUVEC complete growth medium. Cells were seeded in 0.2% gelatin-coated tissues culture flasks and allowed to attach overnight at 37^{o}C in a humidified incubator with an atmosphere of 95% air, 5% CO_2. Medium was replaced the following day to remove erythrocytes and cells were grown to confluence for 2-3 days. Immunofluorescent staining for human von-Willebrand (vWF) factor was employed to confirm the endothelial phenotype of cells. The cells were split 1 in 3 on reaching confluence and experiments were performed on second or third passage HUVEC.

2.3.2. Human aortic endothelial cells (HAEC).

Cryopreserved primary human aortic endothelial cells (HAEC) obtained from Lonza (Slough, UK) were maintained in HUVEC complete M199 medium containing 20% FBS. Cells were split 1 in 3 on reaching confluence and experiments performed on second or third passage.

2.3.3. Bovine aortic endothelial cells (BAEC)

Primary bovine aortic endothelial cells (BAEC) were a gift from Dr Shant Kumar (University of Manchester, UK). Cells were prepared as described by Sattar et al., (1994). BAEC were cultured in RPMI 1640 containing 10% FBS supplemented with GPS and split 1 in 3 on reaching confluence.

2.3.4. Human embryonic kidney 293 cells (HEK 293)

Human embryonic kidney (HEK 293) cells transformed by exposing them to sheared fragments of adenovirus type 5 DNA (Graham et al., 1977). Cells were used to expand adenoviral stocks. HEK 293 were grown in Dulbecco's modified eagle medium (DMEM) containing GPS and 10% FBS. Cells were subcultured 1 in 10-20 once confluent.

2.4. Recombinant Adenoviruses

2.4.1. Amplification and purification of adenoviruses

HEK 293 cells were grown to ~ 70% confluence in DMEM containing 10% FBS and GPS, and then infected with appropriate adenovirus in DMEM containing 2% FBS and GPS. After 2-7 days, when cytopathic effects (medium colour change to yellow and cell detachment) were observed, the supernatant and cells were harvested. The supernatant was aliquoted and stored at -80 ^{o}C. The cell pellet was lysed by 4 cycles of freezing (2-3 mins) in liquid nitrogen and thawing in 37^{o}C water bath (5 mins) to

release their contents. Adenoviruses were purified by equilibrium density gradient centrifugation using caesium chloride gradients prepared as shown in Figure 2.1. Tubes were then centrifuged at 32,000 rpm overnight (12-14 hours). Extracted pure adenovirus (thin white layer located second from the bottom of the tube) was purified by dialysis into optimal buffer (see APPENDIX IV) (Nyberg-Hoffman and Aguilar-Cordova, 1999).

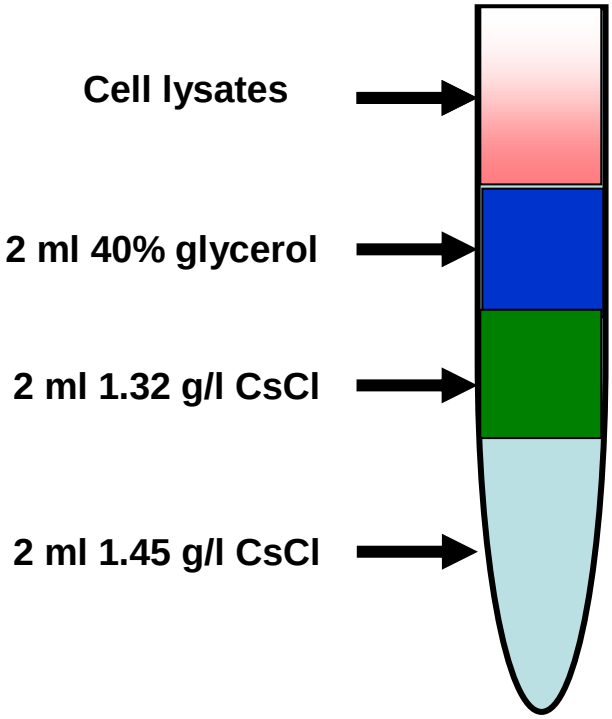


Figure 2.1. Caesium chloride density gradient prepared for adenovirus purification by equilibrium density gradient centrifugation.

2.4.2. Titration of adenoviruses.

Adenovirus titres were determined by infecting $5x10^5$ HEK 293 cpw in 12-well plate with serial dilutions of adenoviral stock (0, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) in 1 ml/well of

DMEM supplemented with 10% FBS and GPS. After 48 hours, the medium was aspirated and cells were fixed immediately with 1 ml of ice-cold 100% methanol. Following a 10 minute incubation at -20^{o}C, cells were rinsed three times with 1 ml of 1% (w/v) bovine serum albumin (BSA) in PBS and 500 µl of mouse anti-Hexon antibody diluted 1:250 in 1% BSA in PBS was added to each well and incubated for 1 hour at 37^{o}C. After another rinsing step, 500 µl of horseradish peroxidise IgG, (HRP-IgG) conjugate diluted 1:500 in 1% BSA in PBS was added to each well, incubated for 1 hour at 37^{o}C and followed by another rinsing step. At this stage, 500 µl of working solution of 3,3'-diaminobenzidine in chromogen solution (DAB) was added to each well and incubated at RT for 10-20 minutes. After the removal of DAB, 1 ml of PBS was added to each well and at least 3 fields of brown/black positive cells were counted using an inverted-phase contrast microscope with 200 x magnification. The number of infectious unit (ifu) per ml was calculated using the following equation.

$$\mathbf{Ifu/ml} = \frac{\text{(Infected cells/Fields) / (Fields/well)}}{\text{Volume virus (ml) X (Dilution factor)}}$$

2.5. Adenoviral infection of HUVEC

HUVEC were grown to ~ 80% confluence before being infected with adenoviruses at a optimal multiplicity of infection (MOI) of 50 (previously determined in-house) for approximately 14 hours in HUVEC growth medium M199 containing 5% FBS. Adenovirus-containing medium was removed and replaced with a fresh MCDB 131 medium containing 10% FBS and GPS and cells were then stimulated in this for the

appropriate time periods. HUVEC require growth factor support and high levels (20%) of FBS for long-term culture. Therefore, to maintain good cell viability during experiments HUVEC were grown in MCDB 131 medium containing 10% FBS, which was originally developed to support the growth of microvascular endothelial cells when supplemented with lower concentrations of serum (Knedler and Ham, 1987). Infection was verified by subjecting protein lysates of infected HUVEC to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Adenovirus	Mutations	Source/Reference
h β-gal	-	(Fujio et al., 1999)
hCMV	CMV promoter without an insert	(Huang and Kontos, 2002)
hPTEN	-	(Huang and Kontos, 2002)
hPTEN-C/S	Cysteine 124 to Serine	(Huang and Kontos, 2002)
hdn-Akt	Serine 473 and Threonine 308 to Alanine	(Fujio et al., 1999)
hmyr-Akt	Myristoylated Akt	(Fujio et al., 1999)
hdn-IκK	Serine 177 and Ser181 to Alanine	(Miyazaki et al., 2000)
mFoxO1-ADA	Serine 256 and 319 to Alanine, Threonine 24 to Aspartic acid	(Nakae et al., 2001)
mVEGF$_{164}$	-	(Bergmann et al., 2010)
mPlGF-2	-	(Luttun et al., 2002)

Table 2.2. Description and source of adenoviruses used in this study. h = human, m = murine.

Western blotting was performed with appropriate antibodies diluted in 5% BSA/0.1% (v/v) Tween^{TM} in tris-buffered saline (TBS-T) (see appendix II for antibody source and dilution used). All adenoviruses used in this study and their source are detailed in Table 2.2.

2.6. Cellular transfections

2.6.1. Small interfering RNA (siRNA) electroporation

RNA interference (RNAi) is a system within living cells that plays a role in controlling gene expression in i.e. development and protection against viral infections. Small interfering RNA (siRNA), which are central to this system, can bind specifically to target gene messenger RNAs (mRNA) and prevent protein synthesis. As shown in Figure 2.2, the RNAi pathway, which is found in many eukaryotes, is triggered in the cell nucleus by the processing of gene-encoded primary microRNA (miRNA) transcripts to miRNA precursors by the RNase-III-like enzyme Drosha. The miRNA precursor is subsequently exported to the cytoplasm where it is further cleaved by Dicer into short fragments of ~ 20 nucleotides, known as siRNAs (duplex-like intermediates). Each siRNA is unwound while incorporating into the RNA-induced silencing complex (RISC). The most well-studied outcome of RNA interference is post-transcriptional gene silencing, which occurs when a mature siRNA strand pairs with a complementary sequence of a mRNA molecule and induces cleavage by argonaute proteins, the catalytic component of the RISC complex (Fire et al., 1998).

HUVEC were electroporated using an AMAXA nucleofector essentially as described by the manufacturer in HUVEC kit I. Fresh MCDB 131 medium containing 10% FBS was pre-warmed to 37^{o}C. Five µl of 100 µM siRNA was used for each condition added in 50 µl of freshly prepared HUVEC nucleofector solution prepared by adding 9 µl of supplement to 41 µl of nucleofector solution and mixed briefly. HUVEC ($2x10^{6}$) were pelleted and resuspended in the nucleofector solution. HUVEC were then transfected with the siRNA using an AMAXA electroporator machine using the A034 programme. The cells were immediately transferred to 1 ml of medium at 37^{o}C for 2 mins before seeding onto 6-well plates and incubated overnight in MCDB 131 containing 10% FBS and GPS. Cells were then stimulated as appropriate.

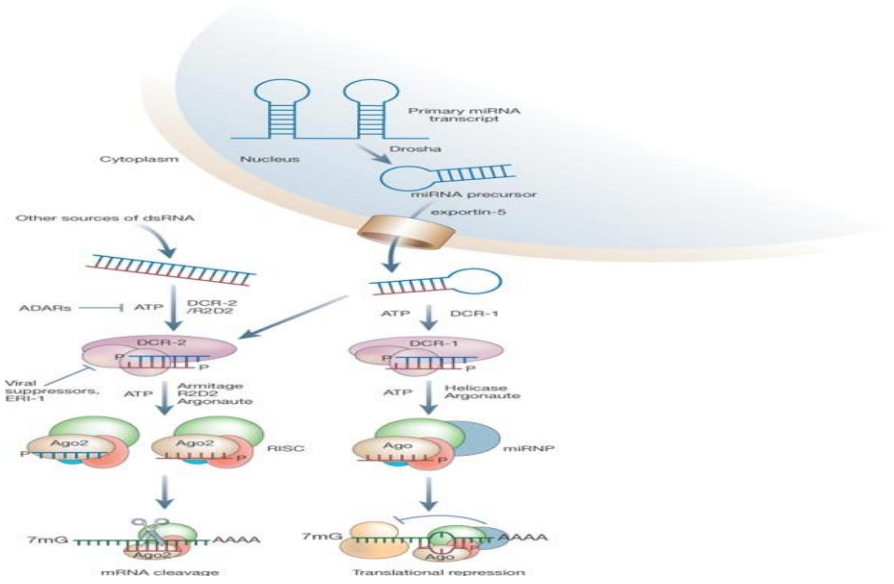


Figure 2.2. Model of small interfering RNA biogenesis and guided post-transcriptional regulation of gene expression. *Primary miRNA are processed to miRNA precursors in the nucleus by Drosha. The miRNA precursor is then processed by Dicer to siRNA duplex-like intermediates assisted by the RNA helicase Armitage and R2D2 in the cytoplasm. The siRNA duplex is then incorporated into RNA-induced silencing complex (RISC). Mature siRNAs repress mRNA translation by binding to argonaute proteins. The stability of the siRNA and its recognition by Dicer can be regulated by specific adenosine deaminases (ADARs) and the exoribonuclease (ERI-1). Taken from (Meister and Tuschl, 2004).*

HEK293 ($2x10^6$) cells were electroporated with siRNA as described above using AMAXA kit V. Cells were pelleted and programme A069 was applied and cells were plated overnight in 1:1 mix of RPMI 1640 and M199 containing 10% FBS and GPS. The medium was then changed to a fresh DMEM containing 10% FBS and GPS and cells stimulated as appropriate. All siRNA sequences used in this study are listed in (Table 2.3), siRNA sequences were either obtained from published literature, or in-house designed using Dharmacon siDESIGN Software (http://www.dharmacon.com/PopUpTemplate.aspx?id=2078) and sequences generated with the highest score were selected and tested empirically.

siRNA	Sequence	Source
siControl	F:5'-UACCCCAUGGCAUUGUCAUtt-3' R:5'-AUGACAAUGCCAUGGGGUAtt-3'	Dharmacon universal control
siPlGF	F:5'-CCGGCUCGUGUAUUUAUUAtt-3' R:5'-UAAUAAAUACACGAGCCGGtt-3'	In-house designed
siVEGF	F:5'-GGAGUACCCUGAUGAGAUCtt-3' R:5'-GAUCUCAUCAGGGUACUCCtt-3'	(Takei et al., 2004)
siVEGFR-1	F:5'-UGAUGGCCUUACACUGAAAtt-3' R:5'-UUUCAGUGUAAGGCCAUCAtt-3'	In-house designed
siVEGFR-2	F:5'-GGAAAUCUCUUGCAAGCUAtt-3' R:5'-UAGCUUGCAAGAGAUUUCCtt-3'	In-house designed
siFOXO1	F:5'-GAGCGUGCCCUACUUCAAGGAtt-3' R:5'-UCCUUGAAGUAGGGCACGCUCtt-3'	(Potente et al., 2005)
siAkt-1	F:5'-GGAGGGUUGGCUGCACAAAtt-3' R:5'-UUUGUGCAGCCAACC CUCCtt-3'	(Jiang et al., 2007)

Table 2.3. Details for siRNA sequences used in this study.

2.6.2. Transfection

BAEC were transfected with plasmids using EX-GEN 500 transfection reagent in RPMI 1640 medium. BAEC cells were plated onto 6-well plates and grown to ~ 70% confluence. Complexes of plasmid DNA and transfection reagent were prepared by mixing and brief vortexing 3 μg of DNA with 10 μl of EX-GEN 500 transfection reagent in 100 μl of medium per well and incubation at RT for 20 mins. The transfection mix was added into each well containing 300 μl medium. After 3 hour incubation at 37^{o}C, 500 μl of medium containing 10% FBS was added and incubated for a further 24 hours. Medium was changed and cells stimulated as appropriate for another 24 hours.

2.7. Gene reporter assay

Luciferase is used as a reporter to assess the transcriptional activity in cells that are transfected with a plasmid construct containing the luciferase gene under the control of the promoter of interest. In luminescent reactions, light is produced by the oxidation of luciferin.

$$\text{luciferin} + O_2 \rightarrow \text{oxyluciferin} + \text{light}$$

Luciferase reporter assays were performed using the Promega dual luciferase reporter assay system according to manufacturer's guidelines. BAEC grown to ~ 80% confluence were co-transfected overnight using EX-GEN 500 with 2 μg of plasmid expressing the firefly luciferase gene under the control of the gene promoter of interest (VEGFR-1) and 50 ng of CMV promoter driven control plasmid expressing the renilla

luciferase gene. After 24 hours, cells were lysed using the luciferase cell lysis buffer, and gene reporter activity was assayed using the Promega dual-luciferase gene reporter assay kit, essentially as described by the manufacturer. Briefly, 10 µl of cell lysate was added to 25 µl of luciferase reagent II in a polycarbonate luminometer tube, the reagents were mixed by brief vortexing and placed in the Berthold luminometer. Luminescence was counted and averaged over 2 periods of 10 seconds. Renilla lucifirase quantification was accomplished by adding 25 µl of Stop & Glow reagent to the same sample tube immediately after quantification of firefly luciferase reaction and luminescence was read again as described above. Luciferase activity in each cell lysate was measured in relative light units (RLU) on a luminometer and Firefly luciferase activity was normalised to Renilla activity for each experiment to control for transfection efficiency.

2.8. RNA extraction

Total RNA was extracted using Norgen cytoplasmic & nuclear RNA purification Kit according to the manufacturer's instructions for cells growing in a monolayer. Cells were washed twice with ice-cold PBS and lysed with 350 µl of lysis buffer per well with gentle tapping and swirling for 5 minutes. Cell lysates were then transferred to a microcentrifuge tube and 200 µl of 95-100% ethanol was added to the lysate. After vortexing for 10 seconds, the lysate was applied onto a mini spin column and centrifuged for 1 minute. The column was washed with 400 µl of washing buffer, centrifuged for 1 minute and the flow through discarded. This step was repeated 3 times. RNA was eluted in 50 µl sterile RNAse-free water. RNA concentrations and purity were estimated using OD 260/280 nm measurement on a spectrophotometer.

2.9. cDNA Synthesis

cDNA synthesis was performed using a Promega reverse transcription kit according to the manufacturer's instructions. 20 µl reactions were prepared by adding the following reagents in the order listed in Table 2.4.

The reaction mix was incubated at 50^{o}C for 1.5 hour. The resultant cDNA was diluted and aliquotted and stored at -80^{o}C for PCR.

Component	Amount	Final concentration
$MgCl_2$ (25 mM)	4µl	5 mM
Reverse transcription 10X buffer	2µl	X1
dNTP mixture (10 mM)	2µl	1mM
RNase inhibitor	0.5µl	-
AMV Reverse transcriptase	15 u (0.6 µl)	-
Oligo($\mathbf{dt})_{15}$ primer, or random primers	0.5µl	-
Total RNA	1µg	-
Nuclease free water to a final volume of	20 µl	-

Table 2.4. cDNA synthesis reaction constituent volumes and concentrations.

2.10. Polymerase chain reaction (PCR)

For standard PCR, a Sunquest thermo-cycler (Arizona, USA) was used for amplification. Biotaq^{TM} DNA polymerase (Bioline Kit) was used following the

manufacturer's instructions. 25 µl reaction mixtures were prepared as described in the Table 2.5. Each reaction was amplified for 35 cycles and PCR products were visualised following agarose gel electrophoresis. For PCR running conditions (see section 2.18.4).

Component	**Volume (µl)**	**Final concentration**
2X Reaction buffer	2.5	1x
$MgCl_2$ (50 mM)	2	4mM
dNTP (100 mM)	1	4mM
DNA Polymerase (5 u/µl)	1	0.2u/µl
Primers (100 µM)	0.5	2 µM
Template cDNA	The equivalent of 2 µg	-
Distilled H_2O	Up to 25	-

Table 2.5. PCR reaction constituents' volumes and concentrations details.

2.11. Agarose gel electrophoresis

DNA products resulting from PCR were visualised in 1 or 2% (w/v) agarose gels electrophoresis. Agarose gels were prepared in 0.5% Tris-borate-EDTA buffer (TBE) with 1 µg/ml ethidium bromide. DNA samples were loaded using 1:5 (v/v) sample to loading buffer. Gels were run at 100 V for approximately 30 minutes and DNA bands were visualised by illumination with UV light at 300 nm.

2.12. Quantitative polymerase chain reaction (qPCR)

SYBR green qPCR was performed using Corbett Rotor-Gene 6000 PCR machine. qPCR reactions were prepared in a total volume of 15 µl using the SensiMix Plus SYBR Kit according to the manufacturer's instructions (see Table 2.6). Primer sequences specific for each target gene are listed in (Table 2.7) were either obtained from published literature, or in-house designed using Primer 3 Sofatware (http://frodo.wi.mit.edu/primer3) and were tested before use ensuring the amplicon is the right size, or by sequencing when necessary. The reactions were performed in triplicate using the following conditions, an initial denaturation step at 95^{o}C for 10 minutes followed by 40 cycles of, denaturation at 94^{o}C for 1 minute; annealing at 60^{o}C for 15 seconds; extension at 72^{o}C for 30 seconds and the fluorescence is read at 78^{o}C after 15 seconds. At the end of the PCR run melt curve analysis was performed between 68^{o}C and 90^{o}C to ensure that a single amplicon was detected. In a real time PCR assay, a positive reaction is detected by accumulation of a fluorescent signal. The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross a defined threshold that exceeds the background level. The threshold was set in the exponential phase of the amplification curve for each reaction. The cycle value for each reaction was established by the cycle number at which each logarithmic PCR plot crossed the threshold line. The Ct value for each gene of interest was normalised using the β-actin Ct value for the same sample. Difference in fold change was then calculated using the equation $2^{-\Delta\Delta ct}$ where ΔΔCt was the ΔCt value from the treated cells minus the ΔCt value for the control.

Component	Volume (µl)
Sensi mix reaction buffer	7.5
$MgCl_2$ (50 mM)	0.6
SYBR green	0.3
Primers (100 µM)	0.06
Template	1
Distilled H_2O	5.54

Table.2.6. qPCR reaction constituents' volumes details.

Gene	Primer Sequence	source
hβ-actin	5'-TCACCCACACTGTGCCCATCTACGA-3' 5'-CAGCGGAACCGCTCATTGCCAATGG-3'	(Malarstig et al., 2003)
mβ-actin	5'-GTATGCCTCGGTCGTACCA-3' 5'-CTTCTGCATCCTGTCAGCAA-3'	In-house designed
hPlGF	5'-GCGATGAGAATCTGCACTGT-3' 5'-CTTTAGGAGCTGCATGGTGA-3'	In-house designed
mPlGF	5'-GAAGTGGAAGTGGTGCCTTT-3' 5'-CGACTCAGAAGGACACAGGA-3'	In-house designed
hVEGFR-1	5'-AGGAGATGCTCCTCCCAAA-3' 5'-GTGCAGGGATCCTCCAAAT-3'	In-house designed
hVEGF	5'-GCCTCGCCTTGCTGCTCTACC-3' 5'-CACACTCCAGGCCCTCGTCATTG-3'	Jayaraman, Padma-Sheela (University of Birmingham, UK)

Table 2.7. qPCR primer sequences. h = human, m = murine.

2.14. Protein biochemistry techniques

2.14.1. Estimation of protein concentration (protein assay)

The amount of protein present in culture medium, or cell protein samples was measured prior to SDS-PAGE using the Bio-Rad protein assay. This colorimetric assay is based on the Lowry protocol and modified to allow estimation of protein concentration in detergents and reducing buffers (Lowry et al., 1951). Protein standards were prepared from a stock solution of 10 mg/ml BSA (w/v) in distilled water and diluted in RIPA buffer to give a range of concentrations from 0.2 to 1.4 mg/ml. Thawed protein samples were diluted 1:5 in RIPA buffer and 5 µl of each sample and the BSA standards aliquotted to duplicate wells (96 well plates). 20 µl of Reagent S added per ml of Reagent A and 25 µl of the mixture added to each well. 200 µl of Reagent B was added to each well and the colour was allowed to develop for 15 minutes and read within 60 minutes in a multiscan ascent 96-well plate reader at 690 nm. Results were obtained by plotting of the BSA standard concentration against optical density (OD).

2.14.2. SDS-PAGE

Equal amounts of protein were diluted in reducing buffer and the samples were heated at 95^{o}C for 10 minutes. Appropriate percentage SDS-polyacrylamide gels (8-15%), dependent on the size of the protein of interest (see Appendix IV). Samples were electrophoresed through the stacking gel at 60 V for 30 minutes and the running gel at 120 V for approximately 2 hours.

2.14.3. Western blotting

After SDS-PAGE, the separated proteins were transferred to nitrocellulose membranes using semi-dry transfer Biorad machine applying 0.8 mA/cm^2 in transfer buffer. Non-specific protein binding on the membrane was blocked by 1 hour incubation with 8% (w/v) fat-free Marvel milk in TBS-T. The membranes were incubated with primary antibody in 5% (w/v) BSA/TBS-T with agitation overnight at 4^oC. After thorough washing with TBS-T, the membranes were incubated with the secondary HRP-conjugated antibody diluted in 8% fat-free milk/TBS-T solution with agitation for 1 hour at RT. After washing with TBS-T, antibody binding was visualised using enhanced chemiluminescent detection (ECL). The band detection process was carried out in the dark room by exposing the membrane to a sheet of autoradiography Kodak film for various time points to achieve the best result for each blot. The films were developed using automated Xograph developer.

2.14.4. Stripping membranes

To re-probe the same membrane with different antibodies, the membrane was stripped by incubation in 30 ml of 0.2 M NaOH at RT for 20 minutes. After washing twice with dH_2O for 5 minutes, the membrane was then blocked again for 30 minutes before incubation with primary antibody.

2.15. PlGF and VEGF Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-linked immunosorbent assay **(ELISA)** for human and mouse PlGF, and VEGF were performed on cell supernatants following the manufacturer's instructions (R&D systems). Each 96-well ELISA microplate was coated using an aliquot of 100 µl of capture antibody diluted in 10 ml of PBS (working concentration) and incubated overnight at RT. The following day, each microplate was washed three times using 250 µl of washing buffer per well. The microplate was then blocked using 250 µl of blocking buffer per well and incubated for two hours at RT. The plate was washed again as described above (3x 250 µl/well). Standard curve was prepared using eight serial dilutions of standard in diluent buffer. Both standards and samples (100 µl/well) were added to the microplate in duplicate and incubated for 2 hours at RT. After another washing step, 100 µl/well of detection antibody diluted in reagent diluent was added and incubated for 2 hours at RT. Following another washing step, 100 µl/well of working dilution of streptavidin-HRP was added and the microplate was incubated for 20 minutes at RT. The plate was washed again, and 100 µl/well of substrate solution was added and incubated at RT for 20 minutes in the dark. The reaction was then stopped by the addition of 50 µl of stop solution. Optical density of each well was measured using a microplate reader at 450 nm and the background at 540 nm subtracted. PlGF or VEGF concentration was determined from the standard curve.

These assays are highly specific and sensitive with reported detection limits of 7 pg/ml for PlGF, and 5 pg/ml for VEGF. The PlGF ELISA shows no cross-reactivity or interference with recombinant human PDGF, $VEGF_{121}$, $VEGF_{165}$ and mouse PlGF-2 prepared at 50 ng/ml. For the VEGF ELISA, R&D systems report that recombinant

human PlGF, VEGF-C, VEGF-D, and mouse PlGF-2, $VEGF_{120}$, $VEGF_{164}$ prepared at 50 ng/ml showed no cross-reactivity or interference with the assay.

2.16. VEGFR-2 ELISA.

A VEGFR-2 sandwich ELISA was developed and optimised in-house by Dr Melissa Cudmore to measure VEGFR-2 in cell lysates. An immobilized capture antibody specific for VEGFR-2 binds both tyrosine-phosphorylated and unphosphorylated

VEGFR-2. After washing away unbound material, a biotinylated detection antibody specific for VEGFR-2 is used to detect captured receptor, utilizing a standard

Streptavidin-HRP format. The assay was performed essentially as described for VEGF and PlGF ELISA. Briefly, 96-well plates were coated with a 100 µl/well of a VEGFR-2 antibody (R&D systems) reconstituted in PBS at a final concentration of 4 µg/ml. For detection, biotinylated anti-VEGFR-2 antibody was reconstituted in reagent diluent buffer at a final concentration of 200 ng/ml and 100 µl/well was added. A VEGFR-2 standard curve was prepared by serial dilutions of recombinant VEGFR-2 in reagent diluent (50-0.781 ng/ml).

2.17. PlGF:VEGF heterodimer ELISA.

The human PlGF:VEGF heterodimer ELISA (R&D systems) was designed to measure human PlGF:VEGF heterodimer in cell-conditioned medium, serum, and plasma. The assay was performed essentially as described for VEGF and PlGF. Briefly, 96-well plates were coated with a 100 µl/well of a PlGF:VEGF heterodimer antibody

reconstituted in PBS at a final concentration of 4 μg/ml/well. For detection, a biotinylated anti-VEGF-A detection antibody (R&D systems) was reconstituted in reagent diluent buffer at a final concentration of 100 ng/ml/well and 100 μl was added. PlGF:VEGF standard curve was prepared by serial dilutions of recombinant PlGF:VEGF in reagent diluent (4000-31.25 pg/ml).

2.18. Chromatin immunoprecipitation (ChIP)

Chromatin Immunoprecipitation (ChIP) is an immunoprecipitation-based experimental technique that is used mainly to investigate the interaction (binding) between DNA and proteins (mapping the DNA target of transcription factors or other chromatin-associated proteins) in the cell. The principle of the technique is outlined in Figure 2.3. Briefly, chromatin bound to DNA in cells is reversibly cross-linked by formaldehyde. The cross-linked chromatin is then sheared by sonication, providing fragments of 200–800 base pairs (bp) in length. Chromatin fragments of 400-500 bp are ideal for for ChIP assays as they encompass two to three nucleosomes. Cell debris in the sheared cell lysate is then cleared by sedimentation and protein–DNA complexes are selectively immunoprecipitated using specific antibodies to the protein/transcription factor of interest of interest. The antibodies are commonly coupled to agarose, sepharose, or more recently magnetic beads. The immunoprecipitated complexes (the bead–antibody–protein–target DNA sequence complex) are then collected and washed to remove non-specifically bound chromatin. The protein–DNA cross-link is reversed and proteins and RNAs are removed by digestion with proteinase-K and RNAse A, respectively. The DNA associated with the complex is then purified and identified by (PCR), microarrays, or direct high-throughput sequencing (ChIP-seq).

To examine the direct binding of FoxO1 to the PlGF and VEGFR-1 gene promoters in endothelial cells, ChIP assay was performed according to manufacturer's instructions on HUVEC grown to ~ 80% confluence in 75 cm^2 flasks. Cells were transduced with HA-tagged, constitutively-active FoxO1 (FoxO1-ADA) and Ad-CMV adenoviral vector with CMV promoter but no insert at (MOI=50) overnight in 12 ml/per flask in HUVEC complete medium containing 5% FBS. The medium was then changed to fresh MCDB 131 medium containing GPS and 10% FBS and incubated for 48 hours at 37 ^oC.

2.18.1. Histone cross-linking to DNA

Histones were cross linked to DNA by adding formaldehyde (final concentration 1% v/v) directly to the culture medium and incubated for 10 minutes at 37^oC. Medium was then aspirated and cells were thoroughly washed twice using ice-cold PBS containing a cocktail of protease inhibitors containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml aprotinin, and 1 µg/ml pepstatin A. After scraping cells into a conical tube, cells were pelleted for 4 minutes at 2000 rpm at 4^oC. Cell pellets were resuspended in warmed SDS lysis buffer containing protease inhibitors cocktail (250 µl of SDS lysis buffer per 1 x 10^6 cells) for 15 minutes before the DNA was sheared.

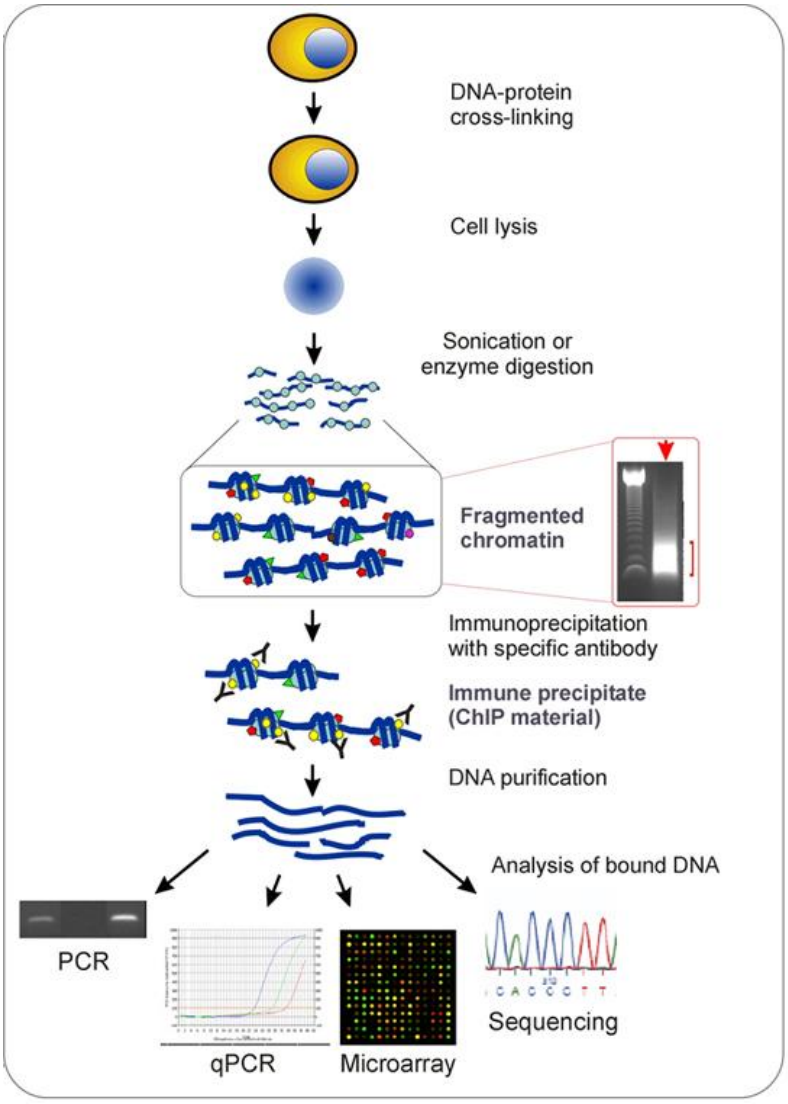


***Figure 2.3. Chromatin immunoprecipitation (ChIP) experimental technique procedure**. Chromatin bound to DNA are cross-linked linked followed by nuclease digestion. The cross-linked chromatin is then sheared by sonication, providing fragments of 200–800 base pairs (bp) in length. protein–DNA complexes are selectively immunoprecipitated using protein-specific antibodies. The immunoprecipitated complexes are then collected and washed to remove non-specifically bound chromatin, the protein–DNA cross-link is reversed and proteins and RNAs are removed by digestion with proteinase-K RNAse A, respectively. The DNA associated with the complex is then purified and identified by (PCR), microarrays, or direct high-throughput sequencing (ChIP-seq). Taken from (Collas and Dahl, 2008).*

2.18.2. DNA shearing/sonication

Cell lysates in SDS lysis buffer were sonicated to shear DNA to lengths between 200 and 800 bp on ice-cold water using Diagenode Bioruptor sonicator. The Bioruptor sonication system, as described by the manufacturer, uses ultrasound waves to create focused mechanical stress to lyse cells, or shear DNA, or chromatin. The ultrasound waves pass through the sample, expanding and contracting liquid. During expansion, negative pressures pull the molecules away from one another and form a cavity or bubble in a process called cavitation. The bubble continues to absorb energy until it can no longer sustain itself and then implodes, producing intense focused shearing forces, which disperses or breaks biomolecules. The fragmentation of DNA takes place as a consequence of this mechanical stress, or shear, from the bubbles. For HUVEC, conditions were optimised as follows. Round 1 for 10 minutes with the power Level set at: high, and ultrasound wave pulse ON for 25 sec, and OFF for 1 min.. Followed by a second round for 7.5 minutes with the power Level set at: high and ultrasound wave pulse ON for 1 mins, and OFF for 25 sec. To ensure the fragments are of the desired size, sheared DNA was reverse cross-linked by adding 8 µl of 5 M NaCl and incubated at 65 ^{o}C for 4 hours followed by DNA extraction by phenol-chloroform method (see section 2.18.4 below). Samples of 5, 10, and 20 µl were run on in 1.5% agarose gel to visualise shearing efficiency and the DNA concentration determined at OD 260/280 on a spectrophotometer for PCR. The remaining DNA was used as 'INPUT'.

2.18.3. Immunoprecipitation

For immunopreciptation, sheared DNA was subjected to further processing steps. Samples were centrifuged for 10 minutes at 13000 rpm at 4^{o}C, the supernatant transferred to a new 2 ml microcentrifuge tube and pellet discarded. Sonicated cell supernatant was diluted 10-fold in ChiP dilution buffer, containing protease inhibitors as above (section 2.18.1). This is done by adding 1800 µl of ChiP dilution buffer to 200 µl sonicated cell supernatant for a final volume of 2 ml in each immunoprecipitation condition. To reduce non-specific binding background, the diluted 2 ml cell supernatant was pre-cleared with 75 µl of protein agarose/salmon sperm DNA (50% slurry) for 30 minutes at 4^{o}C with agitation. The agarose was then pelleted by brief centrifugation and the supernatant collected. This step was followed by adding 5 µg of immunoprecipitation antibody against the HA epitope (YPYDVPDYA) to the 2 ml supernatant fraction and overnight incubation at 4^{o}C with agitation. 60 µl of protein A agarose/salmon sperm DNA (50% slurry) was added for 1 hour at 4^{o}C with rotation to collect the antibody/histone complex. For negative control, a sham immunoprecipitation was performed by incubating the supernatant fraction with 60 µl of protein A agarose/salmon sperm DNA (50% slurry) and no antibody for 1 hour at 4^{o}C with rotation. The agarose was then pelleted by gentle centrifugation (700 to 1000 rpm at 4^{o}C, for 1 min) and the supernatant that contains unbound, non-specific DNA was carefully removed. The protein A agarose/antibody/histone complex was then washed once for 3-5 minutes on a rotating platform with 1 ml of each of the buffers as follows: a) low salt immune complex wash buffer, b) high salt immune complex wash buffer, c) LiCl immune complex wash buffer, and two washes with d) TE buffer. To isolate the DNA that is bound to the immunoprecipitated histone

complex, 250 µl of elution buffer (1% SDS, 0.1 M $NaHCO_3$) was added to agarose/antibody/histone complex from the step above, vortexed briefly to mix and incubated at room temperature for 15 minutes with rotation. The agarose was then removed by centrifugation and supernatant fraction (eluate) was transferred to a new tube. This step was repeated twice, and the resultant eluates (500 µl) combined. This was followed by a reverse cross-linking step and DNA was recovered by phenol-chloroform-isoamyl alcohol (25:24:1) and ethanol precipitation for PCR amplification.

2.18.4. DNA precipitation

The addition of inert carrier, such as glycogen (5 µl of 10 µg/ml) assists the visualisation of DNA pellet. To remove the protein phase, an equal volume of Tris-saturated phenol-chloroform-isoamyl alcohol (25:24:1) was added and the mixture was vortexed and centrifuged for 2 minutes at 12,000 rpm at 4°C. The supernatant was transferred to a fresh tube while avoiding aspiration of interlayer or organic phase. To remove phenol, an equal volume of chloroform was added and the mixture was vortexed and centrifuged for 2 minutes at 12,000 rpm at 4°C. The supernatant was then transferred to fresh tube while avoiding aspiration of interlayer or organic phase. To precipitate DNA, 0.1 volume of 5 M ammonium acetate was added followed by 0.7 volume of isopropanol. The mixture was vortexed and DNA precipitated at -20^{o}C overnight. The precipitate was spun down for 20 minutes at 12,000 rpm 4°C. The supernatant was carefully removed to prevent the loss of the DNA pellet. To wash out salts, 1 ml of cold 70% ethanol was carefully added without vortexing and spun down for 10 minutes at 12,000 rpm 4°C. The pellet was left to air-dry for 10 minutes at RT, but was not allowed to dry completely as it becomes difficult to dissolve. The DNA was

then dissolved in 50 µl of dH_2O, DNA concentration was determined using a spectrophotometer and PCR performed. ChIP primer sequences are in listed in (Table 2.8) were in-house designed using Primer 3 Software and tested prior use for actual experiments (see Section 4.2.9), and PCR was performed in triplicate using the following conditions, an initial denaturation step at 95^{o}C for 5 minutes followed by 40 cycles of, denaturation at 94^{o}C for 1 minute; annealing at 60^{o}C for 30 seconds; extension at 72^{o}C for 1 minute and a final extension step at 72^{o}C for 10 minutes.

gene	Primer sequences	Source
PlGF-seq1	5'-TTG TGT GCA CCT ATG CAA ATC G-'3 5'-CAG TCT GGA CGA CAG AGT AGA AC-3'	In-house designed
PlGF-seq-2	5'-GTC CTT GAT GGT CCT TGA CTT TGC-3' 5'-CAA TGT GTC ATC ACC AAA GGA ATG G-3'	In-house designed
VEGFR-1	5'-GAG TAG CAA GCT GCC ACC AGA AAG-3' 5'-CTT GCT TGG CAC CTT TGG GAA GAC-3'	In-house designed
eNOS	5′-CGGAGCAGGTGATAGAAGCTAGG-3′ 5′-GCTTCCCTGGAGTCTTGTGTAGG-3′	(Potente et al., 2005)

Table 2.8. ChIP primer sequences used in this study.

2.19. Animal experiments

2.19.1. Adenoviral injection of FVB/N mice

FVB/N mice were obtained from Charles River and kindly provided by Dr Andrea Bacon (University of Birmingham, UK) between 8-12 weeks of age weighing between 25-30 g. FVB/N mice were injected *via* the tail vein with $2x10^{10}$ IFU of adenovirus encoding FoxO-ADA, or Ad-CMV empty control virus in a total volume of 100 µl sterile PBS by Mr Ian Ricketts at Birmingham University Biomedical Services Unit. After 48 hours the mice were sacrificied, blood collected to prepare serum, and the livers frozen for the preparation of mRNA and protein extracts.

2.19.2. Ob/ob mouse experiments

The ob/ob mouse tissue and plasma used in this study were obtained through collaboration with Dr Steve Russell (Dept of Pharmacology, Aston University, Birmingham, UK). The Aston ob/ob colony was established in 1966 and has been maintained in a closed non-inbred colony. These mice were originally developed in the Edinburgh Animal Genetics Unit in 1957 using the C57Bl/6J$^{ob/-}$ from Jackson Laboratories and carry the ob gene mutation on a mixed background (JH for greater litter size and CRL for increased growth) which leads to a more severe phenotype than reported for the original and other strains (Bailey et al., 1982). Ob/ob mice and their wild-type (+/+) littermates were sex and age-matched between 16 and 19 weeks old (severly hyperglycaemic) and were housed individually at 20-22^{o}C with 12 hour light:12 hour dark cycles with free access to drinking water and standard chow diet.

2.19.3. Mouse tissue and plasma collection

FVB/N and *ob/ob* mouse liver tissue and serum were collected under the licence of Dr Stuart Egginton (University of Birmingham, Birmingham, UK). Immediately upon collection, tissues were processed as follows

I. Frozen in liquid nitrogen and stored at -80^{o}C until required for Western blot analysis, ELISA or qPCR.
II. Lysed and homogenised in ice-cold 1X RIPA buffer supplemented with protease inhibitors and homogenised using the Precellys tissue homogeniser at a speed of 6,500 x g for 10 minutes.

2.20. Statistical analysis

All data are expressed as mean ± SEM. Statistical comparisons were performed Student's t-test as appropriate. Statistical significance as indicated in figure legends was set at a value of $P < 0.05$.

Chapter 3

The role of the phosphatidylinositol 3-kinase/Akt pathway in the regulation of PlGF secretion from human umbilical vein endothelial cells during acute hyperglycaemia

3.1. Introduction

In humans, PlGF expression is up-regulated in cardiovascular and metabolic disorders characterised by hyperglycaemia and endothelial dysfunction, such as proliferative diabetic retinopathy (Khaliq et al., 1998; Mitamura et al., 2002). Furthermore, elevated levels of PlGF in patients with atherosclerosis is associated with inflammation, vascularisation and plaque instability (Pilarczyk et al., 2008). In animal studies, blocking PlGF or VEGFR-1 inhibited ocular angiogenesis, inflammation and reduced atherosclerotic plaque growth and its susceptibility to rupture in mice (Luttun et al., 2002; Van de Veire et al., 2010). In addition, adenoviral delivery of PlGF-2 into ApoE$^{-/-}$ mice increased intimal thickening and macrophage accumulation compared with the double knockout ApoE$^{-/-}$/PlGF$^{-/-}$ mice, suggesting a role for PlGF in the promotion of atherosclerosis (Khurana et al., 2005).

Signalling through the PI3K/Akt pathway is crucial for metabolic responses to insulin in insulin-responsive tissues and essential for endothelial cell survival, growth and NO generation (Montagnani et al., 2002; Potenza et al., 2005). Hyperglycaemia-induced oxidative stress is thought to be responsible for the down-regulated signalling through this pathway, leading to the development of endothelial dysfunction (Song et al., 2007). In this chapter, the relationship between PlGF expression and the modulation of the PI3K/Akt signalling pathway activity by hyperglycaemia has been examined *in vitro* and *in vivo*.

3.2. Results

3.2.1. Acute hyperglycaemia increases the release of PlGF from HUVEC's *in vitro*.

To determine whether PlGF release from endothelial cells was affected by hyperglycaemia, HUVEC were incubated for 24 hours in growth factor-free MCDB 131 medium containing 10% FBS and normal glucose levels (NG; 5 mM D-glucose), high glucose (HG; 30 mM D-glucose), or osmolarity control (OS; 5 mM D-glucose + 25 mM L-glucose). PlGF levels were measured by ELISA in cell-conditioned medium. Figure 3.2.1 shows that following exposure to high levels of D-glucose the amount of PlGF secreted by HUVEC was approximately 2-fold greater than that secreted by cells maintained in the normal levels of glucose ($p < 0.01$, $n = 4$) and cells that had been treated with an equivalent amount of the physiological inert L-glucose isomer ($p < 0.01$, $n = 4$).

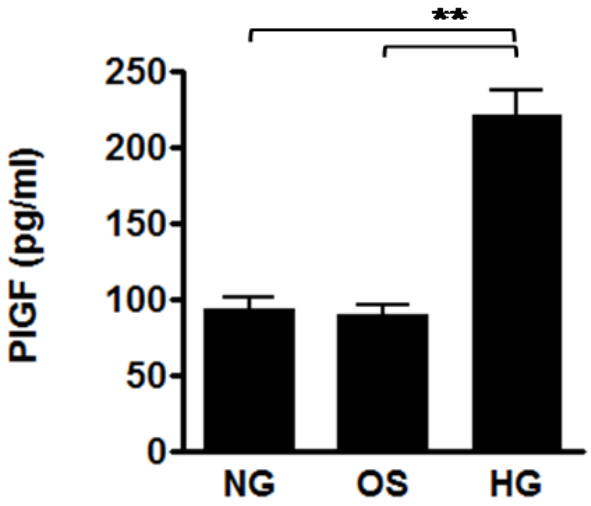


Figure 3.2.1. ***The effect of hyperglycaemia on PlGF secretion in HUVEC****. Cells were cultured for 24 hours in growth factor-free MCDB 131 medium containing 10% FBS and normal glycaemia (NG = 5 mM D-glucose), hyperglycaemia (HG = 30 mM D-glucose) or osmolarity control (OS = 5 mM D-glucose + 25 mM L-glucose). PlGF levels were measured in cell-conditioned medium by ELISA. (**P<0.01, n=4).*

3.2.2. Is VEGF involved in the hyperglycaemia-stimulated PlGF release in HUVEC?

It has been suggested that in bovine retinal microvascular endothelial cells both, the expression of PlGF mRNA and the secretion of PlGF protein can be stimulated during acute periods of hyperglycaemia by an auto- or paracrine mechanism that appears to involve induction of VEGF secretion (Zhao et al., 2004). Therefore, a potential role for VEGF in hyperglycaemia-induced PlGF secretion in HUVECs was investigated using pre-designed short-interfering oligonucleotides (siRNA) sequences directed against the human VEGF gene to 'knockdown' the expression and secretion of VEGF. Effectiveness of the VEGF siRNA was initially tested in a human embryonic kidney cell line (HEK 293) which has a high basal secretion of VEGF. It can be seen in Figures 5.2.6A and 5.2.6B that by following transfection with the VEGF siRNA oligonucleotides the expression of VEGF mRNA was reduced by ~ 50% and the secretion of VEGF protein by > 75 % in HEK 293 cells. A random oligonucleotide siRNA control failed to have any effect on basal VEGF secretion in HEK 293 cells. Basal secretion of VEGF in HUVEC used in this experiment was below the limit of detection of the VEGF ELISA (see Figure 5.2.6C), so it was not possible to directly demonstrate the effectiveness of the VEGF siRNA treatment in HUVEC. However, from the results obtained in the HEK 293 cells, it can be inferred that any hyperglycaemia-stimulated increase in either the expression of VEGF mRNA or secretion of VEGF protein in HUVEC would be significantly reduced following VEGF siRNA treatment.

To test any potential effects of siRNA-mediated knockdowns of VEGF on hyperglycaemia–induced PlGF secretion from HUVEC, cells were transfected with the VEGF siRNA by electroporation as described in the methods section (2.6.1). The effect

of hyperglycaemia on the secretion of PlGF from HUVEC was assessed exactly as described in the experiment shown in section 3.2.1. Figure 3.2.2 shows that there was a significant increase in PlGF secretion under each of the experimental conditions following siRNA-mediated knockdown of VEGF compared to the relevant control ($n = 3$, $p < 0.05$). In 'VEGF-knockdown' cells the effect of hyperglycaemia on PlGF secretion was significantly greater than the additive effect of both treatments on PlGF secretion. The preceding data suggests that it is highly unlikely that acute exposure of HUVEC to a hyperglycaemic stimuli can induce VEGF secretion which then acts in a paracrine fashion to increase PlGF secretion. Since we were unable to verify the knockdown of VEGF in HUVEC, these data should be treated with caution.

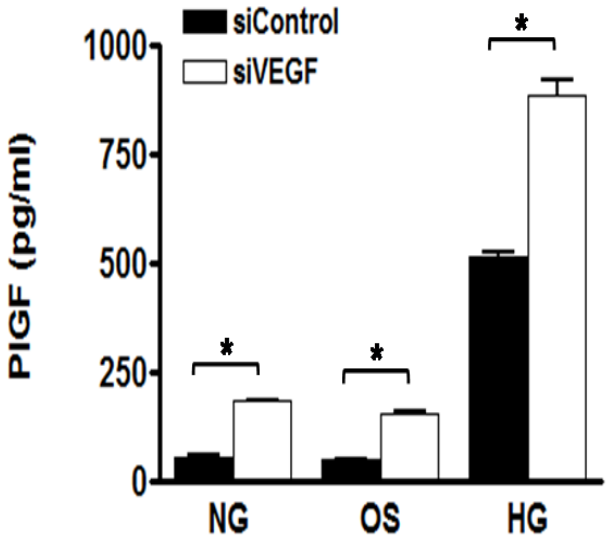


Figure 3.2.2. ***The role of VEGF knockdown in hyperglycaemia-induced PlGF release in HUVEC.*** *HUVEC were electroporated with siRNA targeted to VEGF-A, or control siRNA using an Amaxa nucleofector and incubated overnight. After a further 24 hour incubation in fresh growth factor-free MCDB 131 medium containing 10% FBS and NG, OS or HG PlGF levels were measured in cell-conditioned medium by ELISA. (*P<0.05, n=3).*

3.2.3. IGF-1 reduces PlGF release in HUVEC.

To examine the effect of IGF-1, a classical activator of the PI3K/Akt pathway in endothelial cells *in vitro* and *in vivo* (Song et al., 2007; Sukhanov et al., 2007) on PlGF release, confluent HUVEC were incubated in MCDB 131 medium containing 10 % FBS and increasing concentrations of IGF-1 (0, 0.1, 1, 10, 100 ng/ml) for 24 hours, and PlGF was quantified in cell-conditioned medium by ELISA. As shown in Figure 3.2.3, IGF-1 significantly suppressed PlGF release in HUVEC in a concentration-dependent manner. The effect was maximal at 100 ng/ml, but not significantly different to that observed with 10 ng/ml. Therefore, 10 ng/ml was the standard concentration used for IGF-1 stimulations. These data indicate that activation of the PI3K/Akt pathway may inhibit PlGF release in HUVEC.

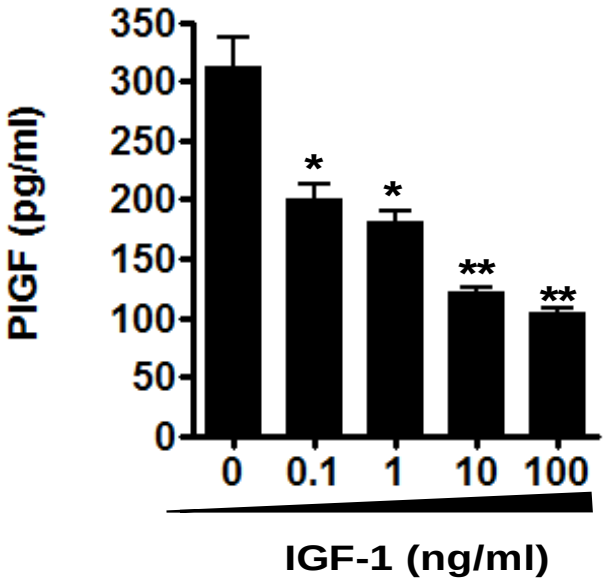


Figure 3.2.3. ***Effect of IGF-I on PlGF release in HUVEC.*** *Confluent HUVEC were stimulated with increasing concentrations of IGF-1 in MCDB 131 medium containing 10% FBS for 24 hours. PlGF concentrations were measured in cell-conditioned medium by ELISA. (*P<0.05, **P<0.01, n=3).*

3.2.4. Combined effects of hyperglycaemia and IGF-1 on PlGF release in endothelial cells.

To determine the combined effects of hyperglycaemia and IGF-1 on PlGF release in endothelial cells, HUVEC and HAEC were incubated in growth factor-free MCDB 131 medium containing 10% FBS and NG, OS or HG in the presence of IGF-1 (10 ng/ml), or vehicle for 24 hours, and PlGF was quantified in cell-conditioned medium by ELISA. The results are shown in Figure 3.2.4A for HUVEC, and Figure 3.2.4B for HAEC. In both cell types, HG, in the absence of IGF-1, significantly induced PlGF release compared with NG and OS. In the presence of IGF-1, this effect was significantly reduced. These data confirm the inductive effect of hyperglycaemia and suppressive effect of IGF-1 on the regulation of PlGF release in endothelial cells.

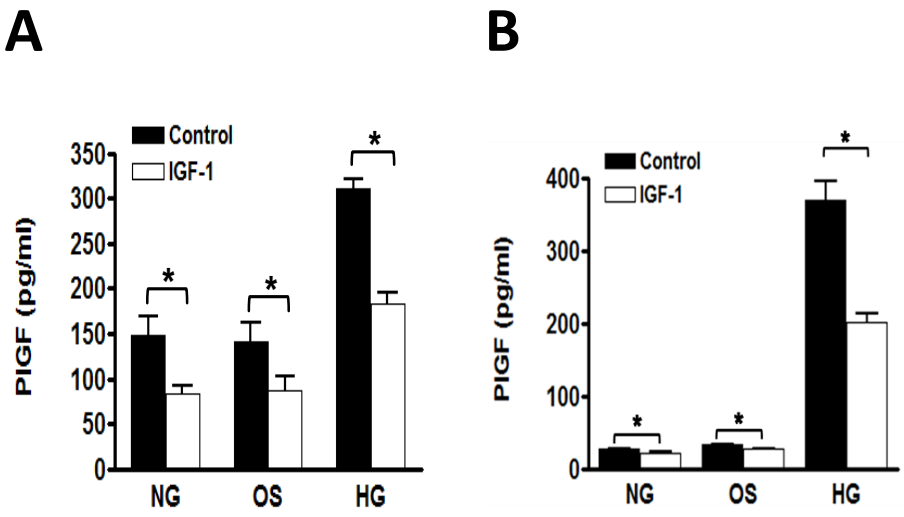


Figure 3.2.4. ***Combined effects of hyperglycaemia and IGF-1 on PlGF release in endothelial cells.*** ***A****, HUVEC and* ***B****, HAEC were cultured in growth factor-free MCDB 131 medium containing 10% FBS and NG, HG or OS control in the presence of IGF-1 (10 ng/ml) or vehicle. After 24 hour incubation PlGF concentrations were measured in cell-conditioned medium by ELISA. (*P<0.05, n=3).*

3.2.5. Combined effect of Hyperglycaemia and IGF-1 on Akt activity in HUVEC.

The suppression of IGF-1-induced Akt activation by hyperglycaemia in HUVEC has been shown by others (Song et al., 2007; Sukhanov et al., 2007). In this section these experiments have been repeated to investigate whether there is a link between Akt phosphorylation status (as a surrogate marker of Akt activity) and PlGF release in glucose stimulated HUVEC. Akt phosphorylation was assessed in cell lysates from HUVEC that had been incubated for 24 hours in growth factor-free MCDB 131 medium containing 10% FBS under normal or hyperglycaemic conditions. At the end of the 24 hour incubation period, cells were stimulated with IGF-1 (10 ng/ml) or vehicle control for 10 minutes and Akt phosphorylation was assessed by Western blotting of cell lysates using an antibody against phospho-Akt ($pAkt^{ser473}$). Western blotting using antibodies recognising Akt and β-actin was performed on stripped and reprobed bltos to determine whether the level of Akt was altered under these conditions and to verify equality of loading respectively.

As shown in Figure 3.2.5, high basal levels of Akt phosphorylation was seen in cells grown under both NG and OS conditions, and the level of Akt phosphorylation was markedly increased by IGF-1 under both conditions. Basal Akt phosphorylation was markedly reduced even under the mildest of hyperglycaemic conditions. IGF-1 was still able to increase the phosphorylation of Akt but the increase in the level of Akt phosphorylation produced by IGF-1 stimulation was much reduced when compared with the relevant controls. Therefore, these results indicate that under conditions of acute hyperglycaemia both basal and agonist stimulated Akt activity is greatly reduced.

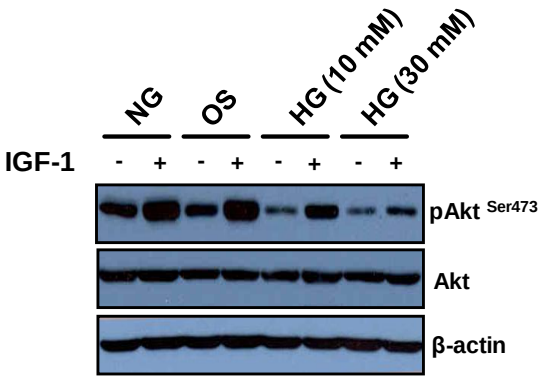


Figure 3.2.5. ***Combined effects of hyperglycaemia and IGF-I on Akt activity in HUVEC.*** *Confluent HUVEC were maintained in medium growth-factor-free MCDB 131 containing 10% FBS and NG, HG or OS control and stimulated with IGF-1 (10 ng/ml), or vehicle for 10 minutes and cell lysates were Western blotted using antibodies recognising phospho-Akt (ser 473), Akt and β-actin. This is a representative blot of 3 independent experiments.*

3.2.6. Effect of inhibition of PI3K by LY294002 on PlGF expression in HUVEC.

To further investigate the role of PI3K in the regulation PlGF expression, HUVEC were incubated with the pharmacological PI3K inhibitor, LY294002 (20 μM). In the experiment depicted in Figure 3.2.6A HUVEC were treated for 8 hours with LY294002 and PlGF mRNA was quantified by qPCR. Under these experimental conditions, LY294002 produce an approximate 2-fold increase in PlGF mRNA expression ($p < 0.05$, $n = 3$, Figure 3.2.6.A). In the second experiment the HUVEC were incubated in the presence or absence of LY294002 for 24 hours. At the end of the incubation period the amount of PlGF protein secreted into the culture medium was quantified by ELISA. Figure 3.2.6B shows that following LY294002 treatment the secretion of PlGF had increased 3-fold relative to control ($p < 0.05$, $n = 3$). The blockade of PI3K by LY294002 was confirmed by probing cell lysates from these experiments with

antibodies recognising pAkt (for activity), Akt and β-actin for equal loading. Figure 3.2.6C clearly shows that the basal level of Akt phosphorylation in HUVEC had been significantly reduced by LY294002. These data present further evidence to suggest that the PI3K signalling pathway may act as a negative regulator of PlGF mRNA expression and protein secretion in HUVEC.

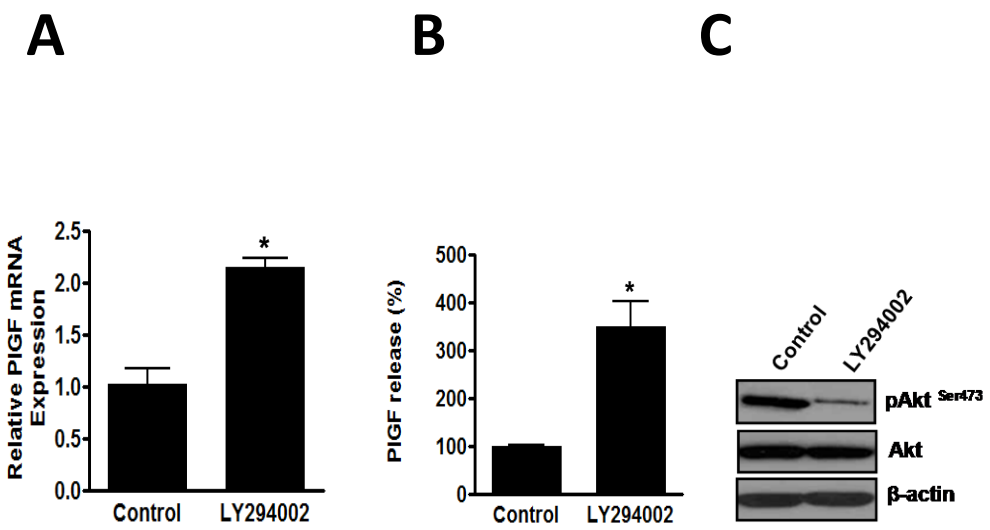


Figure 3.2.6: ***Effect of PI3K inhibition by LY294002 on PlGF expression in HUVEC.*** *Confluent HUVEC were pre-treated with LY294002 (20 μM) and incubated in growth factor-free MCDB 131 medium containing 10% FBS for* ***A****, 8 hours and quantitative PCR (qPCR) was performed on the extracted RNA, or* ***B****, for 24 hours and PlGF protein levels quantified in cell-conditioned medium by ELISA.* ***C****, Cell lysates from B were Western blotted with antibodies recognising phospho-Akt (ser 473), Akt and β-actin.(*P<0.05, n=3).*

3.2.7. Combined effects of LY294002 and hyperglycaemia on PlGF release in HUVEC

To further investigate whether suppression of the PI3K/Akt signalling pathway activity is involved in the regulation of PlGF release, the combined effects of LY294002 and hyperglycaemia on PlGF secretion were examined. The experiment described in section 3.2.1 was repeated in the presence of LY294002 (20 μM), or vehicle. PlGF

secreted into the cell-conditioned medium was quantified by ELISA. As shown in Figure 3.2.7, under normal glucose conditions LY294002 alone, significantly increased the amount of PlGF secreted into the medium bathing the HUVEC by three-four fold when compared with non-treated cells (NG, $p < 0.05$, $n = 3$, OS, $p < 0.05$, $n = 3$). This is also consistent with the results shown in Figure 3.2.6B. As previously shown in Figure 3.2.1, the amount of PlGF secreted into the medium was significantly increased compared to control following exposure to the hyperglycaemic stimuli. However, when this experiment was carried out in the presence of LY294002, there was a further small increase in the amount of PlGF secreted into the medium. However, the amount of PlGF in the conditioned medium in cells that had been both treated with LY294002 and had been exposed to a hyperglycaemic stimuli was not significantly different than that seen in cells that had just been subjected to hyperglycaemia ($n = 3$, $p > 0.05$). This is probably a reflection of the observation that under the conditions used in this experiment the hyperglycaemic stimulus alone was sufficient to almost completely block basal PI3K activity itself (see Figure 3.2.5) and its subsequent stimulatory effects on PlGF secretion was near maximal and inhibition of the remaining residual PI3K activity with LY294002 was only sufficient to increase the extent of PlGF secretion closer to its maximal amount. However, the data still supports a role for PI3K as a negative regulator of PlGF secretion in glucose-stimulated HUVEC.

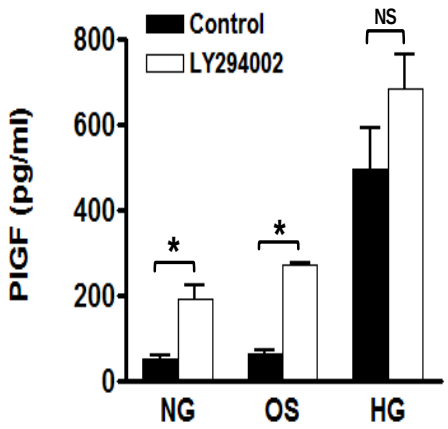


Figure 3.2.7. ***Combined effects of LY294002 and hyperglycaemia on PlGF release in HUVEC.*** *Confluent HUVEC were pre-treated with LY294002 (20 µM), or vehicle and incubated for 24 hours in growth factor-free MCDB 131 medium containing 10% FBS and NG, HG or OS control. PlGF released was measured in cell-conditioned medium by ELISA. (*P<0.05, n=3). NS = not significant.*

3.2.8. Effect of PI3K blockade by PTEN on PlGF release in HUVEC

The experiments with LY294002 support the notion that PI3K is a negative regulator of PlGF expression and secretion in endothelial cells. However, it is well known that pharmacological inhibitors of cell signalling pathways, including LY294002, may have off target effects, especially when used at high concentrations. Therefore, an alternate approach was used to investigate the effects of reducing PI3K activity of PlGF secretion from HUVEC. As discussed in the introductory section, the lipid phosphatase PTEN which acts as negative regulator of the PI3K/Akt signalling pathway by increasing the rate of dephosphorylation of the second messenger PtdIns(3,4,5)P_3 (Maehama and Dixon, 1999). Therefore, activation of the PI3K signalling cascade can be decreased by simply increasing the level of expression of PTEN within cells. This can be achieved practically by adenovirus-mediated over-expression of wild-type PTEN (Huang and

Kontos, 2002). Two controls were used in these experiments, an adenovirus expressing a catalytically inactive mutated PTEN (PTEN-c/s) and an adenovirus encoding a β-galactosidase (β-gal). HUVEC were infected with the adenoviruses containing either, PTEN, PTEN-c/s or β-gal overnight as described in the experimental section. After 24 hour incubation in growth factor-free MCDB 131 medium containing 10% FBS and PlGF in cell-conditioned medium was quantified by ELISA. Adenovirus mediated over-expression of PTEN proteins; functional validation and equal loading were confirmed by Western blotting on cell lysates probed with antibodies recognising PTEN, pAkt, Akt and β-actin (Figure 3.2.8B and C).

As shown in Figure 3.2.8A, over-expression of PTEN significantly induced a 5-fold increase in PlGF release compared with the β-gal over-expressing control ($p < 0.05$, $n = 3$). In Figure 3.2.8C, it can be seen that phosphorylation of Akt is completely abolished by PTEN over-expression. The over-expression of the mutant dominant-negative PTEN-c/s had no effect on PlGF release compared with the β-gal control. These data confirm the role of PI3K in the regulation of PlGF release in HUVEC.

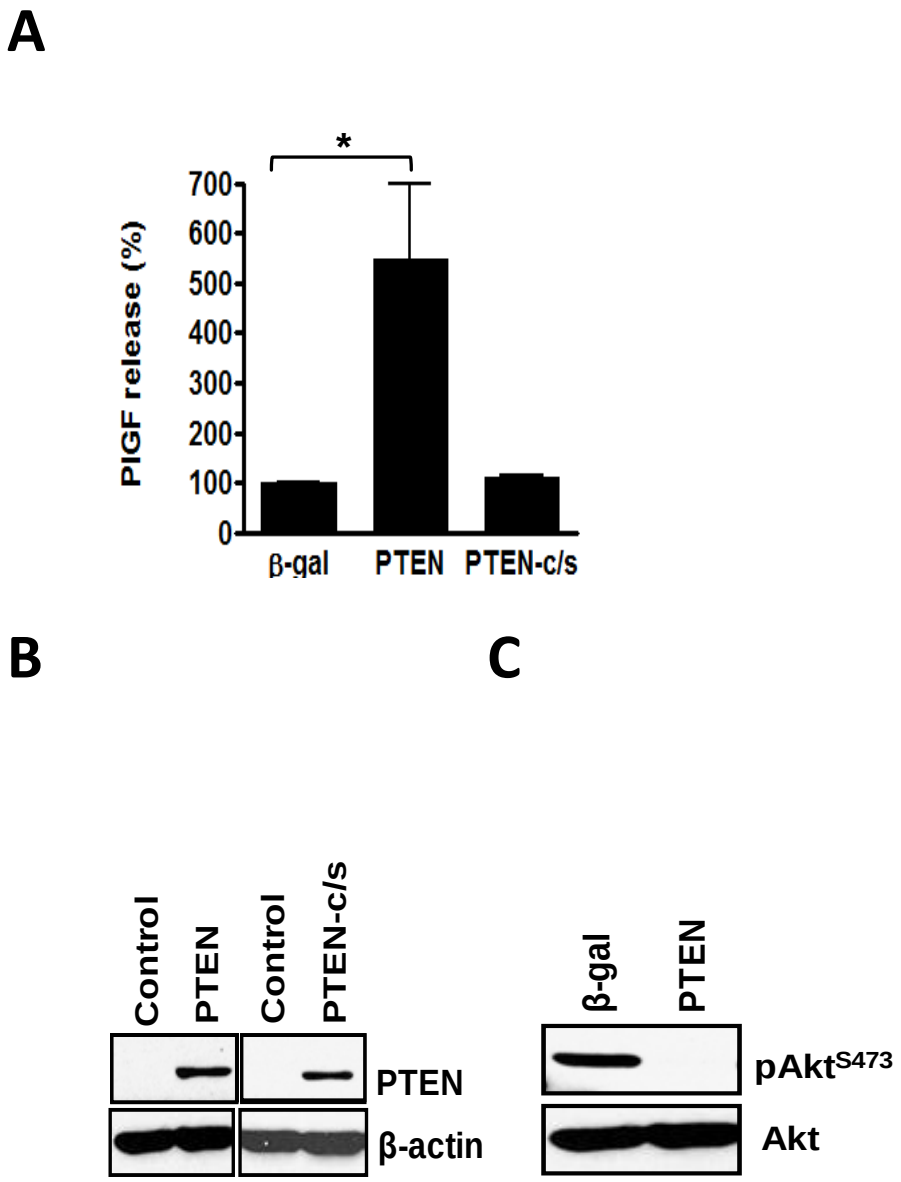


Figure 3.2.8. ***Effect of PTEN expression on PlGF release in HUVEC.*** *Cells were infected overnight with adenoviruses encoding PTEN, dominant-negative PTEN (PTEN-c/s) and β-galactosidase (β-gal). After 24 hour incubation,* ***A****, PlGF in cell-conditioned medium was quantified by ELISA.* ***B*** *and* ***C****, Infection of HUVEC and the status of Akt activity following adenovirus mediated over-expression of PTEN were demonstrated by Western blotting with antibodies recognising PTEN, pAkt, Akt and β-actin. (*P<0.05, n=3).*

3.2.9. Effect of Akt activity modulation on PlGF release in HUVEC

Both pharmacological and enzymatic blockade of PI3K activation increased PlGF release, but it remained unclear which of the PI3K downstream targets is involved in the signalling pathway that controls PlGF release. The serine threonine kinase Akt lies at the top of many of the PI3K activated signalling cascades (Alessi et al., 1997). To examine any potential role of Akt in the regulation of PlGF secretion, adenoviruses encoding mutant Akt were used to manipulate its activity. To this end, HUVEC were infected with adenoviruses encoding either a constitutively-active Akt mutant (myr–Akt) or a dominant-negative Akt mutant (dn-Akt). As a control, HUVEC were infected with an adenovirus encoding β-galactosidase.

Figure 3.2.9A shows the effects of adenovirus over-expression on the degree of phosphorylaytion of Akt on serine 473. It can be quite clearly seen that the level of Akt phosphorylation relative to the β-gal containing adenovirus infected control cells was massively increased in HUVEC infected the myr-Akt containing adenovirus and was significantly reduced in cells infected with the dn-Akt adenovirus.

The experiment depicted in Figure 3.2.9B shows that after an 8 hour incubation with adenovirus containing the dn-Akt construct, PlGF mRNA expression was increased ~ 6-fold relative to the β-gal control ($p < 0.05$, $n = 3$). In contrast, the level of expression of PlGF mRNA in the myr-Akt infected cells was not significantly different from the β-gal control. Figure 3.2.9C shows the amount of PlGF protein in cell-conditioned medium measured over the 24 hour time period after infection with the respective adenoviruses. It can be quite clearly seen that in cells infected with the dn-Akt containing adenovirus the amount of PlGF protein secreted into the medium was reduced by approximately 2-fold when compared with the β-gal control ($p < 0.05$, $n = 3$), whereas, infection with the

myr-Akt containing adenovirus had little effect. This is again evidence for an inhibitory role of the PI3K/Akt signalling pathway in the regulation of PlGF secretion in HUVEC.

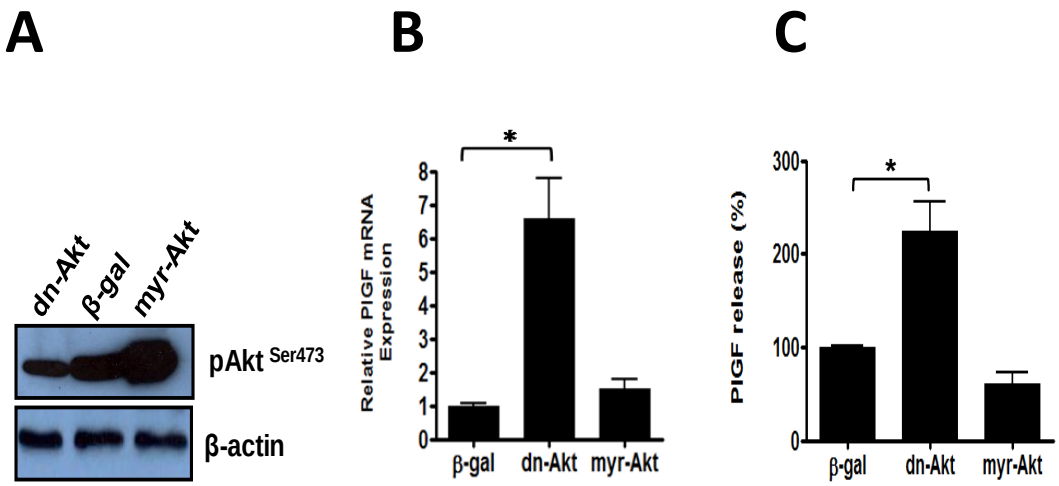


Figure 3.2.9. ***The Effect of Akt activity modulation on PlGF release in HUVEC.*** *HUVEC were infected overnight with adenoviruses encoding dominant-negative Akt (dn-Akt), constitutively-active Akt (myr-Akt) and β-galactosidase (β-gal) in HUVEC complete medium containing 5% FBS. The medium was then replaced with fresh growth factor-free MCDB 131 medium containing 10% FBS.* ***A****, Cell lysates from B were Western blotted using antibodies recognising pAkt (Ser 473) and β-actin.* ***B****, Following an 8 hour incubation qPCR was performed.* ***C,*** *After 24 hour incubation, PlGF levels in cell-conditioned medium were quantified by ELISA. (*P<0.05, n=3).*

3.2.10. Effect of Akt-1 loss on PlGF release in HUVEC.

The experiments described in the previous section suggest that modulation of Akt activity is implicated in the regulation of PlGF expression in HUVEC. To confirm this finding, a complementary approach was sought. Akt-1, the major Akt isoform expressed by endothelial cells (Chen et al., 2005) was silenced in HUVEC by siRNA. The siRNA oligonucleotide pair used to knockdown Akt expression are shown in Table 2.3. Figure 3.2.10A shows the expression of Akt-1 protein was significantly reduced in HUVEC treated with the Akt-1 oligonucleotides siRNA. In Figure 3.2.10B it can be seen in HUVEC treated with the Akt-1 siRNA, the amount of PlGF secreted into the

medium bathing the cells was increased by approximately 3-fold compared with control ($p < 0.05$, $n = 4$). This is another indication that PI3K/Akt signalling pathway plays an important role in the regulation of PlGF release.

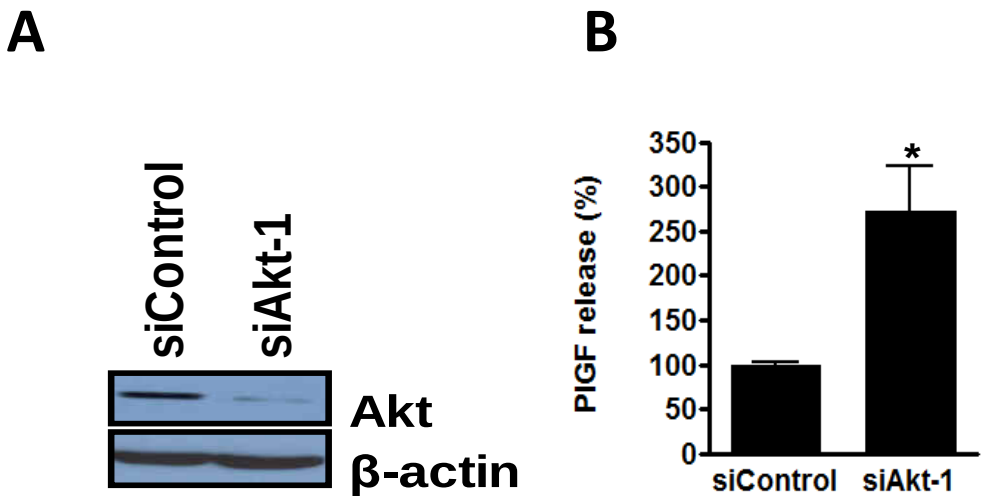


Figure 3.2.10. ***Effect of Akt-1 loss on PlGF release in HUVEC.*** *HUVEC were electroporated with siRNA targeted to Akt-1, or control siRNA using an Amaxa nucleofector and incubated overnight.* ***A****, cell lysates from A were Western blotted using antibodies recognising Akt and β-actin.* ***B****, After further 24 hour incubation in growth factor-free MCDB 131 medium containing 10% FBS, PlGF was measured by ELISA in cell-conditioned medium. (*P<0.05, n=4).*

3.2.11. Is Akt the central regulator of PlGF expression in HUVEC?

To determine whether Akt is the central regulator acting downstream of PI3K, HUVEC were infected with adenoviruses over-expressing PTEN, myr-Akt or β-gal in combination with adenoviruses over-expressing either, PTEN or β-gal. Figure 3.2.11 shows the amount of PlGF protein secreted into the medium over the 24 hour period after infection with the adenoviruses. The right hand panel of Figure 3.2.11 shows that in the cells that had been co-infected with the control β-gal containing adenovirus and the PTEN over-expressing adenovirus that the amount of PlGF protein secreted into the

medium was approximately 2-fold greater than from cells that had been infected with the β-gal control adenovirus alone ($p < 0.05$). In contrast, cells that had been co-infected with β-gal and myr-Akt, the amount of PlGF protein secreted into the medium bathing the cells was approximately 10-fold lower than from cells that had just been infected with the β-gal control adenovirus ($p < 0.01$). In the experiment depicted in the left hand panel of Figure 3.2.11, HUVEC were infected with the PTEN over-expressing adenovirus in combination with either the myr-Akt, PTEN or β-gal containing adenoviruses. It can be clearly seen that the amount of PlGF protein secretion into the medium was increased in cells that had been infected with a combination of the β-gal or PTEN adenoviruses and had received a 'double dose' of the PTEN containing adenovirus when compared with β-gal control. However, the inductive effect of PTEN was still significantly reduced by myr-Akt, indicating that Akt activation overrides the effect of the PI3K inhibition by PTEN. This suggests that Akt is the central downstream effector of PI3K involved in the regulation of PlGF release.

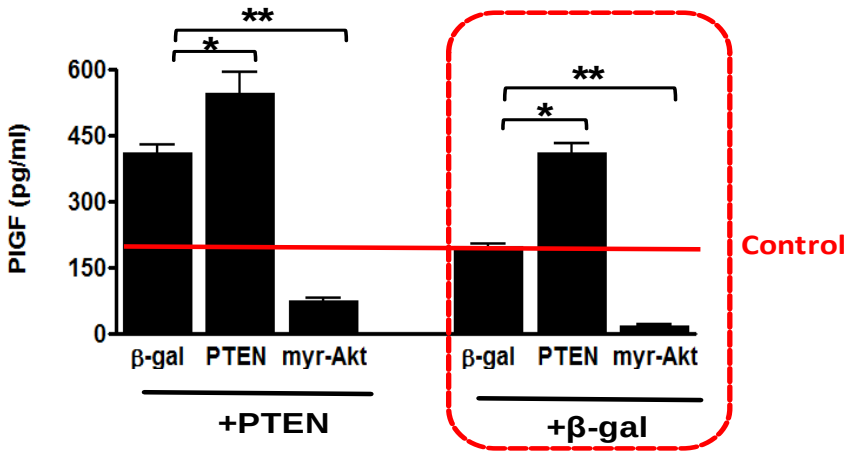


Figure 3.2.11. ***Is Akt the central regulator of PlGF release in HUVEC.*** *HUVEC were co-infected with combinations of PTEN, myr-Akt and β-gal adenoviruses overnight in HUVEC complete medium containing 5% FBS. After a further 24 hour incubation in growth factor-free MCDB 131 medium containing 10% FBS, cell-conditioned medium was collected and PlGF levels quantified by ELISA. (*P<0.05, **P<0.01, n=3).*

3.2.12. Effect of Akt activity on hyperglycaemia-mediated regulation of PlGF expression in HUVEC.

In the next set of experiments the role of Akt in hyperglycaemia-induced PlGF secretion from HUVEC was assessed. Two basic experimental approaches were employed:- 1), assessment of PlGF secretion under basal and HG conditions in HUVEC following siRNA mediated Akt-1 silencing and:- 2), examination of PlGF secretion under basal and HG conditions following over-expression of a constitutively-active Akt. As shown in Figure 3.2.12A, silencing of Akt-1 was sufficient to induce an approximate 2-fold increase in PlGF secretion in cells cultured under normal glucose conditions and in the osmotic control cell cultures. Under HG conditions, PlGF secretion was elevated ~ 7-fold over control and there was no significant additional increase in PlGF release in cells in which Akt-1 expression had been knocked down. This might be due to the fact that under HG conditions near maximal inhibition of the PI3K/Akt signalling pathway had already been achieved and knocking down any residual Akt-1 activity does not have a major impact on PlGF release.

In the second set of experiments, HUVEC were infected with either myr-Akt or β-gal containing adenoviruses and then subjected to hyperglycaemia for 24 hours. Under NG or OS conditions, myr-Akt over-expression had no significant effect on PlGF secretion when compared with the β-gal control.

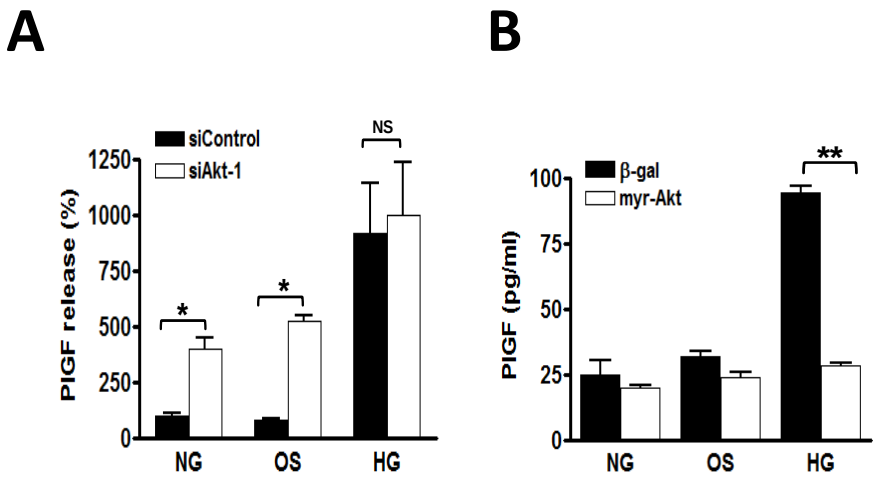


Figure 3.2.12. ***Effect of Akt activity on hyperglycaemia-mediated regulation of PlGF expression in HUVEC.*** ***A,*** *HUVEC were electroporated with siRNA targeted to Akt-1, or control siRNA, using an Amaxa nucleofector and incubated overnight. After further 24 hour incubation in fresh growth factor-free medium containing 10% FBS and NG, OS or HG, and PlGF was measured by ELISA in cell-conditioned medium.* ***B****, HUVEC were infected with myr-Akt or β-gal overnight in HUVEC complete medium containing 5% FBS, followed by 24 hour incubation in growth factor-free MCDB 131 medium containing 10% FBS and NG, OG or HG and PlGF release measured by ELISA. (*P<0.05, n=3). NS = not significant.*

As shown before, PlGF release was significantly increased in cells that over-expressed β-gal control under HG conditions. In contrast, the increase in PlGF secretion produced by exposing the cells to HG conditions was completely abolished by myr-Akt over-expression ($p < 0.05$), suggesting that the suppression of Akt activity is involved in the hyperglycaemia-mediated effects on PlGF secretion (Figure 3.2.12B).

3.2.13. The effect of oxidative stress on PlGF expression in HUVEC.

Hyperglycaemia-induced oxidative stress has been shown to interfere with many cellular processes and is associated with many disorders, in particular, endothelial dysfunction (Song et al., 2007). To determine the effect of oxidative stress on PlGF expression, HUVEC were treated with increasing concentrations of hydrogen peroxide

(H_2O_2; 0, 200, 500, 1000 μM) for 24 hours and PlGF secreted into the tissue culture medium was quantified by ELISA as described in the experimental section. The H_2O_2 concentrations used in this study are widely used in the published literature and had been reported to have no detrimental effect on cell viability provided the experiments were not conducted for longer than 24 hours (Tanaka et al., 2009).

Hydrogen peroxide produced a concentration-dependent increase in PlGF release compared with control, with 500 μM causing maximal effect (Figure 3.2.13A). To investigate whether the effect of H_2O_2 can be reversed by Akt activation, HUVEC were co-treated with 500 μM H_2O_2 and 10 ng/ml IGF-1 for 24 hours and PlGF secreted into the tissue culture medium was quantified by ELISA. As already shown in Figure 3.12.13A, H_2O_2 treatment of HUVEC elevated PlGF secretion compared with untreated cells. The H_2O_2-stimulated increase in PlGF secretion was suppressed by approximately 30% in the presence of IGF-1 ($p < 0.01$). This is very reminiscent of the effect IGF-1 had on the hyperglycaemia-induced secretion of PlGF (Figure 3.2.4). At best these results show that oxidative stress-induced PlGF secretion can be reversed by activation of the Akt signalling pathway. Perhaps, these results suggest a potential role for Akt in oxidative stress up-regulation of PlGF expression, but further experiments will have to be performed to prove this conjecture.

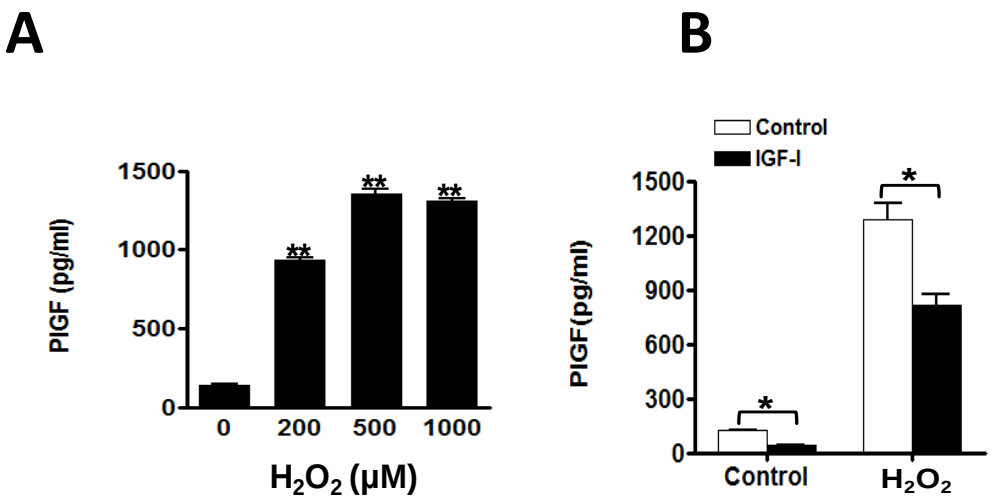
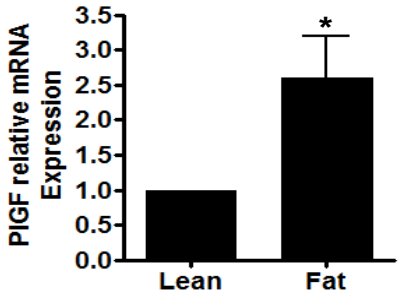


Figure 3.2.13. The effect of oxidative stress on PlGF expression in HUVEC*. Confluent HUVEC treated with,* ***A****, increasing concentrations of H_2O_2 and* ***B****, 500 µM H_2O_2 with IGF-1 (10 ng/ml), or vehicle for 24 hours. PlGF levels were quantified by ELISA in cell-conditioned medium. (*P<0.05, **P<0.01, n=3).*

3.2.14. PlGF levels in obese *ob/ob* mice compared with their lean littermates.

To confirm the role of PI3K/Akt pathway in the regulation of PlGF release *in vivo*, PlGF mRNA and protein were quantified in the severely hyperglycaemic obese *ob/ob* mouse model. Obese ***ob/ob*** mice are reported to show suppressed PI3K/Akt activity in the muscle and liver tissue compared with their lean littermates (Folli et al., 1993; Kerouz et al., 1997). As shown in Figure 3.2.14A, PlGF mRNA levels quantified by qPCR in liver tissues were significantly higher in obese mice compared with their lean littermates. However, PlGF protein levels measured by ELISA in tissue homogenates, although higher in the *ob/ob* mice compared with the lean littermates, did not reach statistical significance (Figure 3.2.14B). This is possibly due to the fact that PlGF is secreted and not retained within the cells. PlGF protein in plasma was significantly higher in the fat mice compared with the control, as shown in (Figure 3.2.14C).

A



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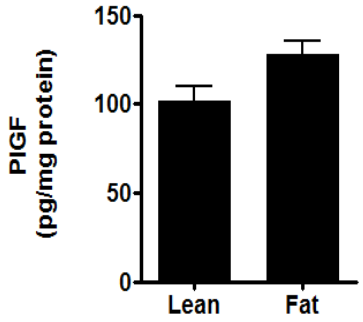

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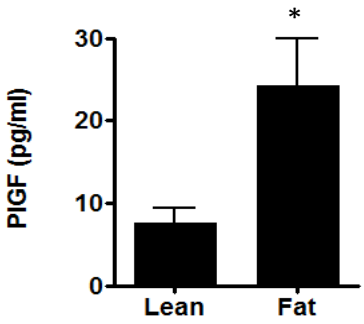


Figure 3.2.14. ***PlGF levels in obese ob/ob mice compared to their lean littermates.*** ***A,*** *Livers were homogenised in RIPA buffer, RNA extracted and PlGF mRNA quantified by qPCR. PlGF was measured in* ***B****, liver tissue extracts and* ***C****, in plasma by ELISA. (*P<0.05, n=6).*

3.3. Discussion

In this study the role of the PI3K/Akt signalling pathway in the regulation of PlGF expression by acute hyperglycaemia was examined in endothelial cells. In HUVEC, hyperglycaemia induced PlGF protein levels over 24 hours compared with control. This is in agreement with findings of Boulton and colleagues (Zhao et al., 2004) who examined the relationship between PlGF mRNA and protein expression and increasing concentrations of D-glucose (10 mM, 15 mM, 20 mM and 25 mM) in bovine retinal microvascular endothelial cells. Although they showed an increase in PlGF mRNA and protein expression in response to HG compared with control by semi quantitative RT-PCR and Western blotting, my findings differ, because 30 mM D-glucose, which is the most commonly used glucose concentration *in vitro* and *in vivo* studies (Du et al., 2000), increased PlGF protein release significantly compared with control 5 mM D-glucose. Whereas, they found that 15 mM D-glucose significantly induced PlGF mRNA and protein. Concentrations lower or higher or than 15 mM D-glucose had no significant effect (to either control, or 15 mM respectively). The difference could be because controls for osmolarity were not used in the Zhao study. The effect of hyperglycaemia on PlGF expression in endothelial cells, as the data in this study suggest, is PI3K/Akt-dependent. Additionally, Zhao and colleagues suggested that hyperglycaemia-induced PlGF release is secondary to endogenous VEGF, but they did not show the presence of VEGF in the endothelial cell type used, its up-regulation by hyperglycaemia, or the loss of response to hyperglycaemia when VEGF was knocked down.

In humans, the pathological role of PlGF in the promotion of inflammation, vascularisation in atherosclerotic plaques (Pilarczyk et al., 2008) and diabetic patients

with proliferative retinopathy (Khaliq et al., 1998; Mitamura et al., 2002) have been shown. In animal studies, the role of PlGF in the progression of atherosclerosis in atherosclerosis susceptible ($ApoE^{-/-}$) mice has also been documented (Khurana et al., 2005; Luttun et al., 2002). Moreover, the loss or inhibition of PlGF and/or its receptor, VEGFR-1, in mice inhibited the aforementioned disorders (Khaliq et al., 1998; Mitamura et al., 2002). In agreement with these findings, in this study it was shown that IGF-1, which was previously reported to inhibit the progression of atherosclerosis in $ApoE^{-/-}$ mice through the suppression of inflammation and oxidative stress generation (Sukhanov et al., 2007) significantly reduced PlGF expression in a concentration-dependent manner in cultured HUVEC.

In adipocytes, skeletal muscle and hepatocytes, the activation of the PI3K/Akt predominantly mediates the metabolic effects of insulin via the promotion of glucose up-take through the translocation of GLUT4 to cell membrane (James et al., 1988; James et al., 1989). The disruption of this signalling pathway is therefore considered the main cause of insulin resistance in metabolic tissues and the development of diabetes. In endothelial cells, which also express both insulin and IGF-1 receptors, but not GLUT4, insulin and IGF-1 function as mitogens, promote endothelial cell survival (Montagnani et al., 2002), and phosphorylate eNOS to promote NO production (Zeng et al., 2000; Zeng and Quon, 1996). Hyperglycaemia-induced impairment of PI3K/Akt signalling in diabetes was found to abrogate NO production leading to the up-regulation of pro-inflammatory adhesion molecules such as endothelin-1 (ET-1), vascular cell adhesion molecule 1 (VCAM-1), E-selectin (Min et al., 2005; Potenza et al., 2009a; Potenza et al., 2009b; Quagliaro et al., 2005), generation of reactive oxygen species (ROS) (Du et al., 2000; Esposito et al., 2002; Quagliaro et al., 2003) and induction of

endothelial cell apoptosis (Baumgartner-Parzer et al., 1995; Du et al., 1998; Lorenzi et al., 1985). These alterations culminate in endothelial dysfunction.

In this study, 24 hour exposure of endothelial cells to 30 mM D-glucose increased PlGF secretion significantly compared with control, and IGF-1 suppression of this effect was accompanied by changes in Akt phosphorylation status. Changes in PlGF protein were accompanied by even greater increases in mRNA levels. The inhibition of Akt by hyperglycaemia induced PlGF release, and conversely, its activation by IGF-1 suppressed it, consistent with the interpretation that possibly the same mechanism that causes endothelial dysfunction, induces PlGF release in endothelial cells in diabetes. To dissect the role of the PI3K/Akt pathway in the modulation of PlGF release, complementary approaches were used. Inhibition of PI3K using the pharmacological inhibitor, LY294002, or adenovirus-mediated over-expression of PTEN significantly increased PlGF release at an mRNA and protein level compared with control. The inductive effect of LY294002 alone on PlGF expression was not significantly different from that in the presence of HG. These data implicate PI3K in the regulation of PlGF expression and confirm that HG effect suppresses PI3K activity in HUVEC. This finding is in agreement with studies showing PTEN up-regulation by hyperglycaemia in HUVEC, mediated by the tumour suppressor transcription factor LKB1 (Song et al., 2007). In another study, the specific inhibition of PTEN expression in mice reversed these effects of hyperglycaemia (Butler et al., 2002). Oxidative stress induced by hyperglycaemia, also suppresses Akt activity (Song et al., 2007) and is considered the underlying cause of endothelial dysfunction in diabetes. To evaluate whether oxidative stress plays any role in PlGF regulation, HUVEC treated with increasing concentrations of H_2O_2 showed significantly elevated PlGF levels (almost 10-fold) compared with non-

treated cells, and the suppression of this inductive effect by IGF-1 suggested that PlGF up-regulation by oxidative stress is may be PI3K/Akt-mediated and that it may be a stress response, lending further support to the hypothesis implicating PlGF in the promotion of inflammation in endothelial dysfunction. Examination of oxidative stress effect on PlGF expression in the presence and absence of Akt activity in endothelial cells would answer the question whether Akt is the main mediator of this effect.

To explore which of the PI3K downstream effectors control PlGF release, Akt activity was manipulated and PlGF release was measured. Inhibition of Akt activity using dominant-negative Akt (dn-Akt) adenovirus in the absence of HG significantly induced PlGF expression at an mRNA and protein level. In contrast, adenovirus-mediated over-expression of constitutively-active Akt (myr-Akt) suppressed it, or had no effect compared with control. In the presence of hyperglycaemia, the HG-promoting effect on PlGF expression was markedly blunted by constitutively-active Akt. To confirm this finding, an alternative approach was used. Akt-1 knockdown in HUVEC alone, in the absence of hyperglycaemia significantly induced PlGF expression compared with control. Since the inductive effect of Akt-1 loss under basal conditions was not significantly different from that in the presence of hyperglycaemia, this suggested that hyperglycaemia effect is Akt-mediated. This is in line with the data of Fernandez-Hernando and colleagues (Fernandez-Hernando et al., 2007) who showed by gene array, although not confirmed by qPCR, that Akt-1 knockdown in $ApoE^{-/-}$ mice fed a Western diet, showed 3-fold greater PlGF expression. These mice lacking Akt-1, exhibited exacerbated atherosclerosis and occlusive coronary artery disease.

VEGF up-regulation has been implicated in hyperglycaemia-mediated pathologies (Aiello et al., 1994). VEGF promotes angiogenesis by promoting PlGF expression in endothelial cells (Carmeliet et al., 2001), and PlGF up-regulation by hyperglycaemia was suggested to be secondary to VEGF induction (Zhao et al., 2004). To differentiate whether the observed effects on PlGF expression by hyperglycaemia are secondary to increased VEGF levels, or to the suppression of the PI3K/Akt pathway, siRNA to VEGF-A, or control siRNA were used to block its synthesis under NG, OS and HG conditions. VEGF knockdown in the absence of HG increased PlGF expression under all conditions compared with control, possibly representing a mechanism to restore homeostasis. Under hyperglycaemia conditions, levels of PlGF released in the absence of VEGF increased further, suggesting that increased PlGF release induced by hyperglycaemia is independent of VEGF.

The hypothesis linking PI3K/Akt to PlGF expression regulation by HG was confirmed by *in vivo* data showing PlGF levels are significantly higher both at mRNA and protein levels in the liver and plasma of the severely obese and hyperglycaemic ob/ob mice compared with their lean littermates. One can speculate that hyperglycaemia-induced up-regulation of PlGF expression may contribute to the development of cardiovascular disorders, retinopathy and possibly obesity in diabetic patients.

Taken together, this study suggests that the PI3K/Akt signalling pathway is a central regulator of PlGF expression by hyperglycaemia in endothelial cells. Understanding how PlGF expression is regulated may provide novel targets for therapy for pathologies characterised by endothelial dysfunction and inflammation.

Chapter 4

Examination of transcription factors downstream of Akt activation regulating PlGF and VEGFR-1 expression in endothelial cells.

4.1. Introduction

In Chapter 3, the PI3K/Akt signalling pathway was shown to play a negative role in PlGF expression in endothelial cells, but little is known of the transcriptional regulation of PlGF expression downstream of Akt. A number of possible Akt effectors, including mTOR, NF-κB and forkhead transcription factors, have been identified (Manning and Cantley, 2007). Little is known about the promoter region of the PlGF gene but one report suggests that one or more NF-κB binding sites are present in the PlGF promoter (Cramer et al., 2005). In insulin-responsive tissues, the most abundant of the forkhead transcription factors is FOXO1. FOXO1 has been identified as the primary downstream target of PI3K/Akt activation by insulin in the maintenance of glucose homeostasis (Accili and Arden, 2004). In endothelial cells, FOXO1 activation mimics the cytotoxic and pro-inflammatory effects of hyperglycaemia and oxidative stress that have been implicated in the promotion of atherosclerotic plaque formation and rupture through the induction of LDL oxidation (Potente et al., 2005; Tanaka et al., 2009), but whether FOXO1 is involved in the up-regulation of PlGF expression that accompanies these diseases has not been addressed.

The involvement of VEGFR-1 in pathologies characterised by inflammation and vascularisation, such as cancer, arthritis, atherosclerosis and choroidal neovascularisation, has been documented (Kami et al., 2008; Luttun et al., 2002; Murakami et al., 2006). Transcriptional regulation of VEGFR-1 was previously shown to be inhibited by nuclear factor of activated T cells (NFAT) (Jinnin et al., 2008), and enhanced by early growth response factor-1 (Egr-1) (Vidal et al., 2000). Gene array analysis following either loss or gain of FoxO1 function in HUVEC suggests that

FOXO1 plays an important role in the regulation of VEGFR-1 expression (Daly et al., 2004; Potente et al., 2005). In this chapter, the role of NF-κB and FOXO1 in the regulation of PlGF and VEGFR-1 expression in human endothelial cells under basal conditions and in hyperglycaemia were investigated.

4.2. Results

4.2.1. The role of NF-κB in the regulation of PlGF expression in HUVEC.

It has been suggested that NF-κB mediates cellular processes downstream of Akt in endothelial cells .i.e. cell cytotoxicity (Zhou et al., 2008). In addition, NF-κB activity induction by hyperglycaemia have been shown in endothelia cells, leading to increased expression of pro-inflammatory molecules and induced cell apoptosis, two key triggers of atherosclerosis (Morigi et al., 1998; Sheu et al., 2005) . Furthermore, binding sites for NF-κB on the PlGF gene promoter have been identified and shown to be functional in fibroblasts (Cramer et al., 2005). In pursuit of identifying effectors downstream of Akt implicated in the regulation of PlGF expression in endothelial cells, NF-κB, seemed to be an ideal target to investigate first. To this end, NF-κB activity was inhibited and the effect on PlGF protein expression was quantified. Under the experiment conditions depicted in the Figure 4.2.1A, HUVEC incubation with isohelenin (1μM), a highly specific and potent pharmacological inhibitor of NF-κB activation (Lyss et al., 1998), had no significant effect on PlGF expression compared with control ($p > 0.05$, $n = 3$). Since pharmacological inhibitors of cells signalling may have off target effects, an alternative approach to inhibit NF-κB activity was sought. Under normal physiological conditions, NF-κB proteins are sequestered in an inactive state in the cytoplasm by

dimerisation with a member of the endogenous inhibitory protein family IκB (Inhibitor of κB) (Gilmore, 2006). NF-κB activation is triggered by the signal-induced degradation of IκB proteins. This occurs primarily via the phosphorylation of two serine residues on the regulatory domain of IκB mediated by a kinase called the IκB kinase (IKK) which targets IκB for ubiquitinylation and destruction in the proteosome. Once activated the NF-κB molecule translocates to the nucleus where it is involved in regulation of the expression target genes. Therefore, an alternative way of blocking of NF-κB activation in HUVEC is infection with an adenovirus encoding an IKK mutant (dn-IKK) that cannot phosphorylate IκB so preventing it degradation. As shown in Figure 4.2.1B, the amount of PlGF released in the cell-conditioned medium after 24 hour incubation was not altered in dn-IKK-infected cells compared with control ($p > 0.05$, $n = 3$), again, suggesting that NF-κB does not play a role in the regulation of PlGF expression in endothelial cells. However, since we have not actually demonstrated activation of NF-κB by hyperglycaemia in our hands, this data has to be interpreted with some caution.

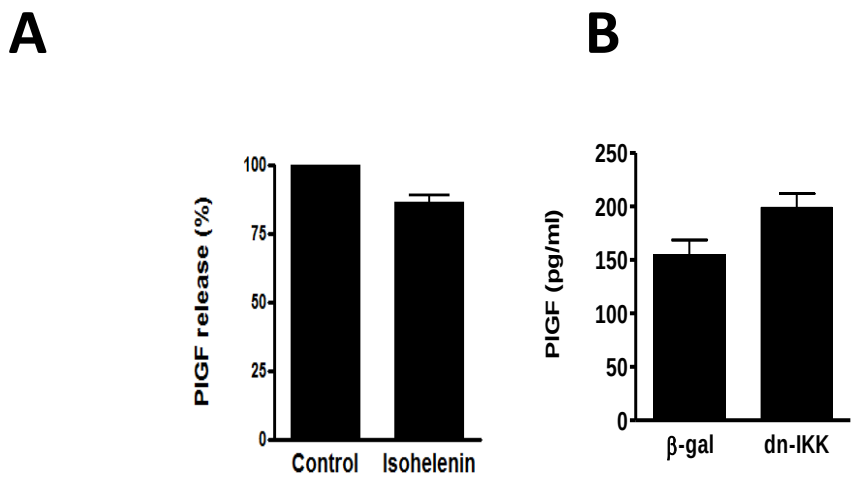


Figure 4.2.1. The role of NF-κB in the regulation of PlGF expression in HUVEC. *PlGF was quantified by ELISA in cell-conditioned medium from,* ***A****, Confluent HUVEC treated with isohelenin (1 μM), or control for 24 hours.* ***B****, HUVEC transduced with dominant-negative IKK (dn-IKK), or β-gal adenoviruses overnight followed by 24 hour incubation in growth factor-free MCDB 131 medium containing 10% FBS. (n=3).*

4.2.2. Effect of modulating Akt activity on FOXO1 phosphorylation in HUVEC.

The identification of FOXO1 as the primary downstream target of PI3K/Akt activation by insulin in the maintenance of glucose homeostasis in metabolic tissues (Accili and Arden, 2004) and the mediation of hyperglycaemia and oxidative stress in the promotion of atherosclerotic plaque formation and rupture through the induction of LDL oxidation (Potente et al., 2005; Tanaka et al., 2009), warranted the examination of whether FOXO1 is involved in the up-regulation of PlGF expression that accompanies these disorders. To demonstrate that FOXO1 is downstream effector of Akt in endothelial cells, HUVEC were infected with recombinant adenoviruses encoding constitutively-active myristoylated Akt (myr-Akt), dominant-negative Akt (dn-Akt), or β-gal and their effects on FOXO1 phosphorylation, a surrogate marker of FOXO1 activity, were examined by Western blotting as described in the Methods (section 2.5). Cell lysates were Western blotted using antibodies recognising phospho-FOXO1 (Serine 256), suggested that constitutively-active Akt induced FOXO1 phosporylation, whereas, there was a slight decrease in dn-Akt-infected HUVEC compared with β-gal. Blots were also probed with antibodies recognising FOXO1 and β-actin to verify change in FOXO1 expression and equal loading (Figure 4.2.2).

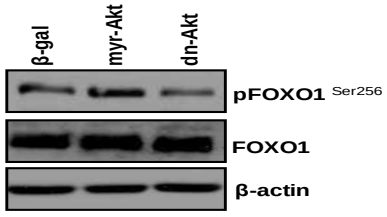


Figure 4.2.2. Regulation of FOXO1 phosphorylation by expression of mutant Akt in HUVEC. *Confluent HUVEC were transduced with adenoviruses encoding β-gal, constitutively–active Akt (myr-Akt) and dominant-negative Akt (dn-Akt) overnight in HUVEC complete medium containing 5% FBS followed by 24 hour incubation in growth factor-free MCDB 131 medium containing 10% FBS. Cell lysates were Western blotted using antibodies recognising phospho-FOXO1 (Ser 256), FOXO1 and β-actin. This is a representative blot of 3 independent experiments.*

4.2.3. Effect of hyperglycaemia on FOXO1 phosphorylation in HUVEC.

In Chapter 3, it was shown that hyperglycaemia abrogated Akt phosphorylation in HUVEC, in agreement with previously published literature (Song et al., 2007). To further determine that FOXO1 is acting downstream of Akt and whether the suppression of Akt activity by hyperglycaemia had any effect on the phosphorylation status of FOXO1 activity, HUVEC were exposed to normal and high levels of hyperglycaemia (NG, OS or HG) in the presence of IGF-1 (10 ng/ml), or vehicle, as described in section 3.2.5. Cell lysates were probed with antibodies recognising phospho-FOXO1 (Serine 256) and the results are shown in Figure 4.2.3. In the absence of IGF-1, it was difficult to assess the effect of HG (10 and 30 mM D-glucose) on FOXO1 activity since FOXO1 phosphorylation levels were very low under all conditions. However, IGF-1 strongly induced FOXO1 phosphorylation under all conditions (NG, OS and HG). This stimulatory effect on FOXO1 phosphorylation was, however, largely eliminated by HG (30 mM D-glucose) compared with NG and OS, whereas, moderate HG (10 mM D-glucose) had very little, if any effect. These data indicate that the downstream target of Akt FOXO1 is activated by hyperglycaemia in HUVEC. Expression of FOXO1 and β-actin were also examined to demonstrate equal loading using antibodies recognising FOXO1 and β-actin (Figure 4.2.3).

4.2.4. Effect of FOXO1 knockdown on PlGF expression in HUVEC.

In the experiments described in the previous section, it has been shown that FOXO1 is a downstream effector of Akt that is activated by hyperglycaemia, but whether FOXO1 plays any role in the regulation of PlGF expression is still not known. To address this question HUVEC were transduced with siRNA duplex to inhibit FOXO1

protein synthesis, or control siRNA duplex as described in the Methods (section 2.6.1). FOXO1 knockdown was verified by Western blot analysis on cell lysates using antibodies recognising FOXO1, and β-actin to confirm equal loading as shown in Figure 4.2.4A. FOXO1 knockdown significantly decreased PlGF protein secreted in cell-conditioned medium by ~ 50% compared with control ($p < 0.05$, $n = 3$), these results indicate that FOXO1 is implicated in the regulation of PlGF expression (Figure 4.2.4B).

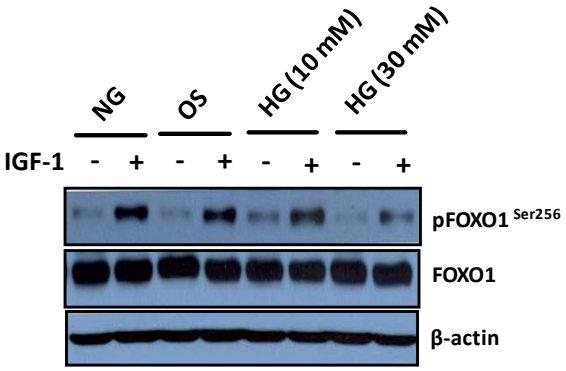


Figure 4.2.3. ***Effect of hyperglycaemia and IGF-1 on FOXO1 phosphorylation in HUVEC.*** *Confluent HUVEC were maintained growth factor-free MCDB 131 medium containing 10% FBS and NG, HG or OS and then stimulated with IGF-1 (10 ng/ml) or vehicle for 10 minutes. Cell lysates were Western blotted using antibodies recognising phospho-FOXO1 (Ser 256), FOXO1 and β-actin. This is a representative blot of 3 independent experiments.*

To confirm that FOXO1 is acting downstream of PI3K/Akt to regulate PlGF expression, the experiment in 4.2.4B was repeated, but in the presence of the PI3K pharmacological inhibitor, LY294002 (20 μM). Following 24 hour incubation, PlGF protein was quantified in cell-conditioned medium by ELISA. The results in Figure 4.2.4C, indicate that inhibition of the PI3K/Akt signalling pathway in the presence of FOXO1, as expected, significantly increased PlGF expression compared with control. This induction was, however, abolished when FOXO1 was knocked down ($p < 0.05$, n

= 3), suggesting that PI3K/Akt regulation of PlGF expression is FOXO1-mediated and that FOXO1 is acting downstream of the PI3K/Akt.

The identification of the essential role of FOXO1 in the regulation of PlGF expression warranted the examination of its effect on the hyperglycaemia-mediated induction of PlGF expression. To this end, FOXO1 was silenced in HUVEC overnight, this was followed by 24 hour incubation in normal, or hyperglycaemia (NG, OS or HG), essentially as described in Section 3.2.12. Figure 4.2.4D indicates that HG, as expected, markedly augmented PlGF release compared with NG, OS in the presence of FOXO1. The silencing of FOXO1, however, significantly abolished the inductive effect of HG compared with control ($p < 0.05$, $n = 3$). These data, again, confirm a role for FOXO1 as an effector of the PI3K/Akt in the regulation of PlGF expression by hyperglycaemia.

4.2.5. Effect of FOXO1 gain-of-function on PlGF expression in HUVEC.

The observed effect of FOXO1 loss-of-function raised the question whether FOXO1 gain-of-function would have the converse effect on PlGF expression. To examine this hypothesis, HUVEC were transduced with recombinant adenoviruses expressing constitutively-active FoxO1 (FoxO1-ADA), this adenovirus expresses a mutated FOXO1 protein that cannot be phosphorylated by active Akt, hence, preventing its expulsion from the nucleus (see Table 2.2)), or β-gal overnight in HUVEC as described in Section 2.5, followed by a further 24 hour incubation in the presence of IGF-1 (10 ng/ml), or vehicle. PlGF secreted in cell-conditioned medium was measured by ELISA. Figure 4.2.5A demonstrates the over-expression of FoxO1-ADA compared to non-infected cells as confirmed by Western blot analysis on cell lysates using antibodies

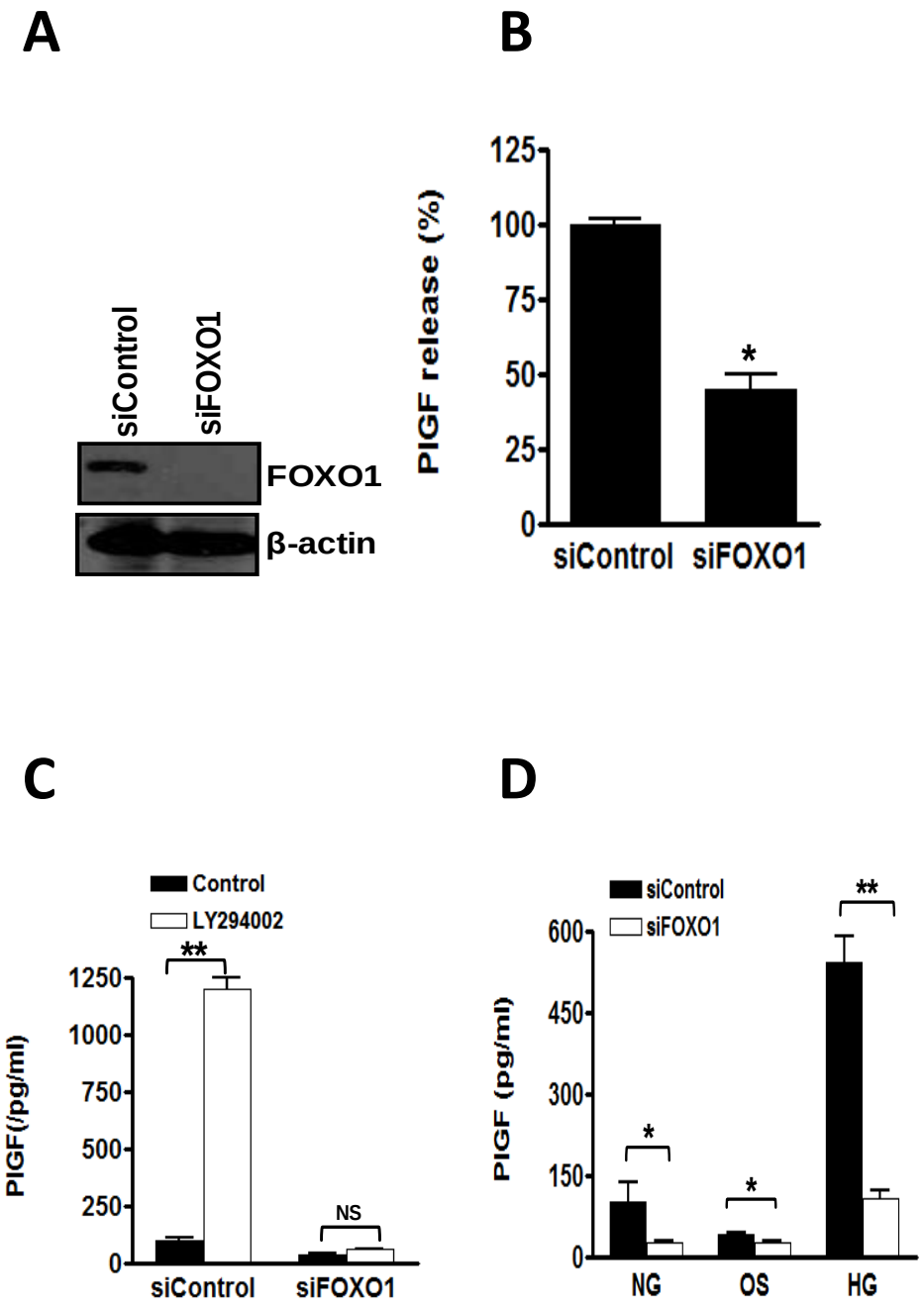


Figure 4.2.4. ***Effect of FOXO1 knockdown on PlGF release in HUVEC.*** *HUVEC were electroporated with siRNA targeted to FOXO1 or control using an Amaxa nucleofector and incubated overnight.* ***A****, After 24 hour incubation in growth factor-free MCDB 131 medium containing 10% FBS, PlGF was measured by ELISA in cell-conditioned medium.* ***B****, Cell lysates from A were Western blotted with antibodies recognising FOXO1 and β-actin.* ***C****, Cells were treated with LY294002 (20 μM) or control for 24 hours and PlGF was quantified in cell-conditioned medium by ELISA.* ***D****, Cells were incubated for 24 hours in growth factor-free MCDB 131 medium containing 10% FBS and NG, OS or HG, and PlGF levels were measured by ELISA in cell-conditioned-medium. (*P<0.05, **P<0.01, n=3).*

recognising β-actin and HA tag on FoxO1-ADA adenovirus. As shown in Figure 5.2.5B, there was a significant induction (~ 4-fold) of PlGF expression in FoxO1-ADA-transduced HUVEC over control in the presence and absence of IGF-1. However, the activation of the PI3K/Akt pathway and phosphorylation of FOXO1 by IGF-1 in the presence of β-gal significantly suppressed PlGF expression. In contrast, this suppressive effect was largely eliminated in the presence of FoxO1-ADA. These experiments further confirm that PI3K/Akt effects on PlGF expression in HUVEC are FOXO1-dependent (Figure 4.2.5B).

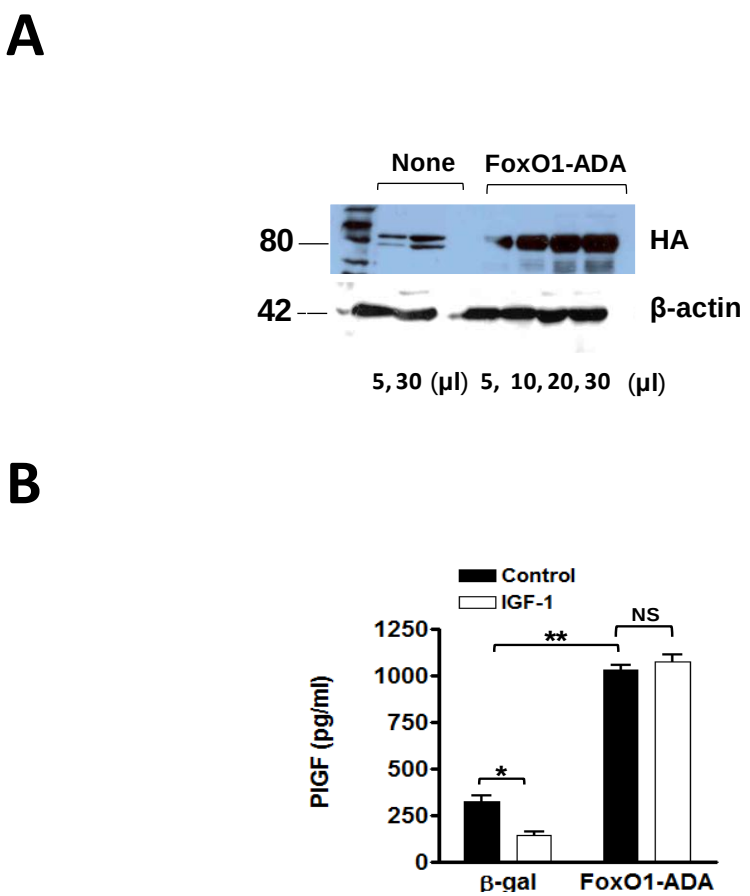


Figure 4.2.5. Effect of FOXO1 gain-of-function on PlGF expression in HUVEC. *HUVEC were transduced overnight with adenoviruses encoding constitutively-active FoxO1 (FoxO1-ADA) or β-gal in HUVEC complete medium containing 5% FBS.* ***A****, After 24 hour incubation in growth factor-free MCDB 131 medium containing 10% FBS in the presence of IGF-1 (10 ng/ml) or vehicle, PlGF was quantified in cell-conditioned medium by ELISA.* ***B****, Increasing volumes of cell lysates of FoxO1-ADA-infected and non-infected HUVEC were Western blotted using antibodies recognising the HA epitope and β-actin. (*P<0.05, **P<0.01, n=3). NS = not significant.*

4.2.6. FOXO1 gain-of-function effect on PlGF expression *in vivo*.

Although the role of FOXO1 in the regulation of PlGF expression *in vitro* was shown in the preceding section (4.2.5), the demonstration of this role *in vivo* has not been previously demonstrated. To examine the role of FOXO1 in the regulation PlGF expression *in vivo*, FVB/N mice were injected with 2 x 10^{10} ifu of recombinant adenoviruses expressing FoxO1-ADA, or a control adenovirus (Ad-CMV) via the tail vein (i.v). A further 48-60 hours was allowed for the virus to propagate and produce its biological effects. The mice were then sacrificed and the expression of PlGF mRNA in the liver was quantified and PlGF protein measured in the serum.

The expression of PlGF mRNA following adenoviral administration to mice was investigated in the liver as it is well established that the adenoviruses delivered i.v. are rapidly filtered by the liver leading to a strong expression of the insert in this tissue (Matsumoto et al., 2006). In addition, previous studies had demonstrated the dual role of FoxO1-ADA in the regulation of hepatic insulin sensitivity and lipid metabolism in liver 5 days after the i.v. delivery of 4×10^9 ifu of the same adenovirus in C57BL/6 male mice (Matsumoto et al., 2006), which provided us with a starting point to optimise the adenoviral dose. The injection of FVB/N with (4×10^{10}) FoxO1-ADA was lethal 24 hours post injection; therefore, it was reduced to the abovementioned concentration. PlGF mRNA in the liver ($p < 0.05$, $n = 8$) and protein ($p < 0.05$, $n = 10$) levels in serum were significantly higher in FoxO1-ADA-injected mice compared with Ad-CMV-infected controls (Figure 4.2.6A and 6B), thus confirming our *in vitro* results.

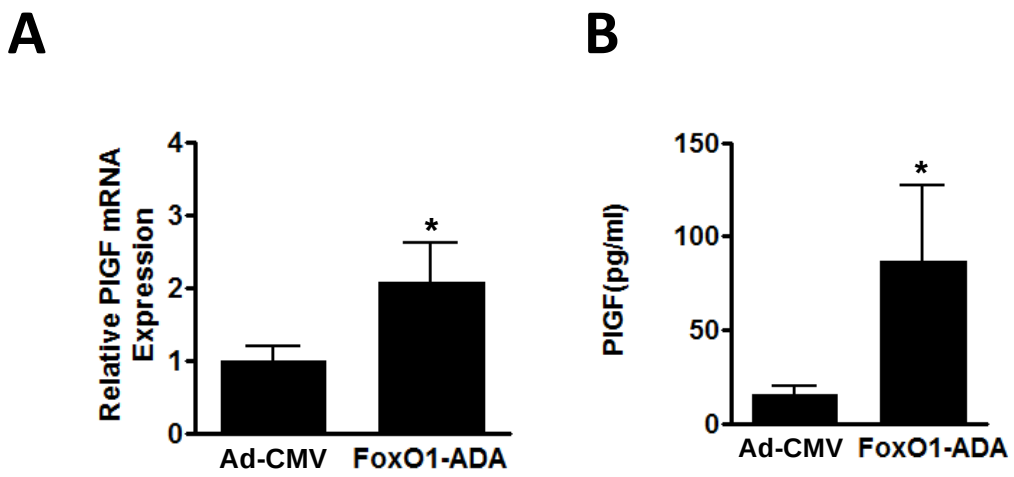


Figure 4.2.6. ***Effect of FoxO1 gain-of-function on PlGF expression in vivo****. FVB/N mice were transduced with adenoviruses encoding FoxO1-ADA or Ad-CMV for 48-60 hours, and* ***A****, livers homogenised, RNA extracted and PlGF mRNA quantified by qPCR (n=8).* ***B****, PlGF protein levels quantified in plasma by ELISA (n=10). (*P<0.05).*

4.2.7. Does FOXO1 interact with the PlGF and VEGFR-1 gene promoters?

To examine the direct binding of FoxO1 to the PlGF and VEGFR-1 gene promoters in endothelial cells, ChIP assay were performed. To identify potential FOXO1 binding sites on the PlGF gene promoter, conserved regions in the 5' region of the PlGF gene were identified by alignment of 10 Kb sequences immediately upstream of the start codon from human, chimpanzee, marmoset, bovine, pig, rat and mouse using the Vista web-based search engine (http://genome.lbl.gov/vista/mvista). To identify conserved putative FOXO1 transcription factor binding sites within these sequences the human PlGF sequence was subjected to transcription factor binding site searches using a combination of TFsearch (http://www.cbrc.jp/research/db/TFSEARCH.html) and TESS (http://www.cbil.upenn.edu/cgi-bin/tess) search engines in regions of high homology. Two promising conserved regions of the PlGF gene which contained putative FoxO binding sites were identified. PCR primers were designed to amplify these regions for

ChIP assays. PlGF ChIP site 1 (PlGF-seq 1) is ~ 3.6 Kb and ChIP site 2 (PlGF-seq 2) is ~ 5.7 Kb upstream of the start codon. The PCR product sizes were 252 bp (-5716 to –5968 bp), and 184 bp (-3613 to -3797 bp) for ChIP sites 1 and 2 respectively (see Figure 4.2.7).

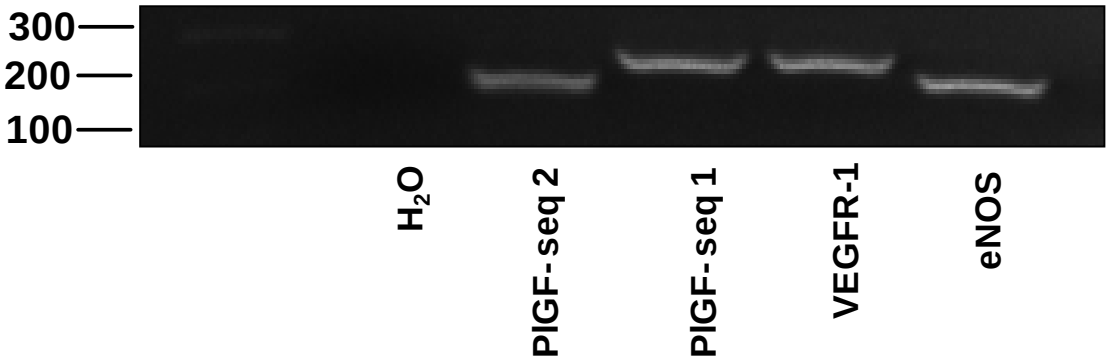


Figure 4.2.7. ***PCR amplification of fragments of PlGF, VEGFR-1 and eNOS genes.*** *PCR optimisation for PlGF-seq 1 and 2, VEGFR-1 and eNOS ChIP primers with HUVEC sheared genomic DNA used as a template.*

PlGF-seq 1 contains a 5'-GATTTGTT-3' a FoxO binding site as described by Gao X *et al*., (2010), 5'-TTGTTTTG-3' and other potential FoxO binding motifs containing 5'-xxTTTGTT-3' and 5'-TTGTTTxx-3'. PlGF-seq 2 contains an inverse FOXO response element 5'-CTAAACA-3' and two novel Forkhead binding motifs, 5'-GACAAG-3' and 5'-GACATG-3', that were recently described by, (Gao et al., 2010) immediately flanking the site.

4.2.8. Chromatin-bound DNA shearing optimisation

To shear chromatin-bound DNA to the optimal fragment size (200-800 bp), briefly, HUVEC chromatin was cross-linked to DNA using HUVEC complete medium containing 1% (v/v) 37% formaldehyde for 10 minutes at 37^{o}C. Chromatin-bound DNA was sonicated, the DNA was then purified and DNA fragments were run on 1.5% agarose gel. As shown in Figure 4.2.8, DNA fragmentation was optimised to successfully yield 200-800 bp fragments. (See Methods Section 2.18.2).

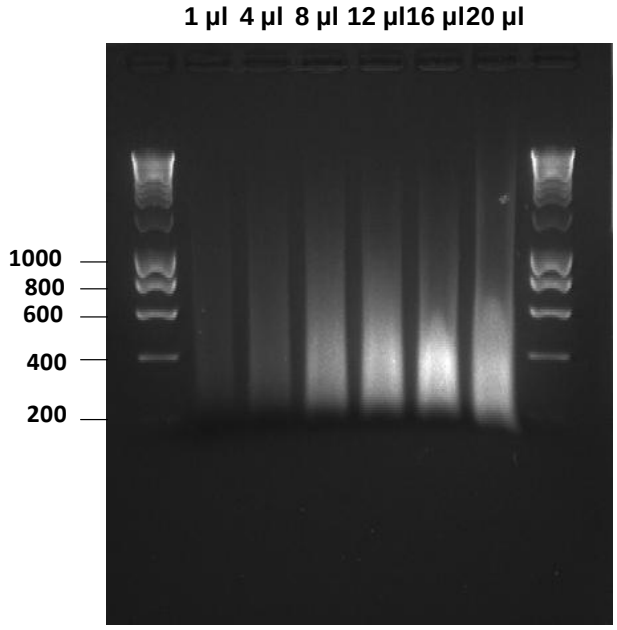


Figure 4.2.8. ***DNA fragmentation****. Confluent HUVEC fixed in HUVEC complete medium containing 1% (v/v) 37% formaldehyde for 10 minutes, cells were then lysed, DNA was sheared and increasing volumes of purified DNA fragments were run on 1.5% agarose gel. This is a representative gel of 3 independent experiments.*

4.2.9. Optimisation of PCR conditions for ChIP primers on human genomic DNA from HUVEC.

To optimise PCR conditions for PlGF and VEGFR-1 ChIP primers, PCR analysis was performed on purified sheared DNA from HUVEC using primers flanking predicted FOXO1 putative binding sites on the PlGF and VEGFR-1 promoters using transcription factor binding site search engines (see details below). Figure 4.2.7 shows PCR amplification of two fragments containing putative FOXO1 binding sites in the PlGF gene promoter, referred to as PlGF–seq 1, PlGF-seq 2, a binding site on the VEGFR-1 promoter and a previously reported FOXO1 binding site on the eNOS gene promoter (positive control) located at -2765 bp upstream the start codon (Potente et al., 2005). These data indicate that HUVEC DNA shearing and ChIP primers are working.

4.2.10. The ChIP analysis of PlGF gene promoter.

To determine whether FOXO1 directly binds to the PlGF gene promoter at these putative binding sites, ChIP analysis was performed on HUVEC transduced with FoxO1-ADA, Ad-CMV adenoviruses, or non-infected cells for 48 hours. Chromatin-bound DNA was immunoprecipitated with an antibody against the HA epitope (HA-tagged FOXO1-ADA). Immunoprecipitated DNA was then analysed by PCR using primers designed flanking the binding the putative sites (PlGF-seq 1 and PlGF-seq 2) PCR amplification of genomic DNA fragments using PlGF-seq 1 and PlGF-seq 2 and Input DNA that had not been immunoprecipitated serve as a positive control (4.2.9A and B).

PCR amplification of immunoprecipitated HA-tagged DNA fragments from FoxO1-ADA-infected cells indicates that FOXO1 directly binds to the PlGF promoter at PlGF-

seq 1, but not PlGF-seq 2. A plasmid encoding PlGF was used as an additional positive control.

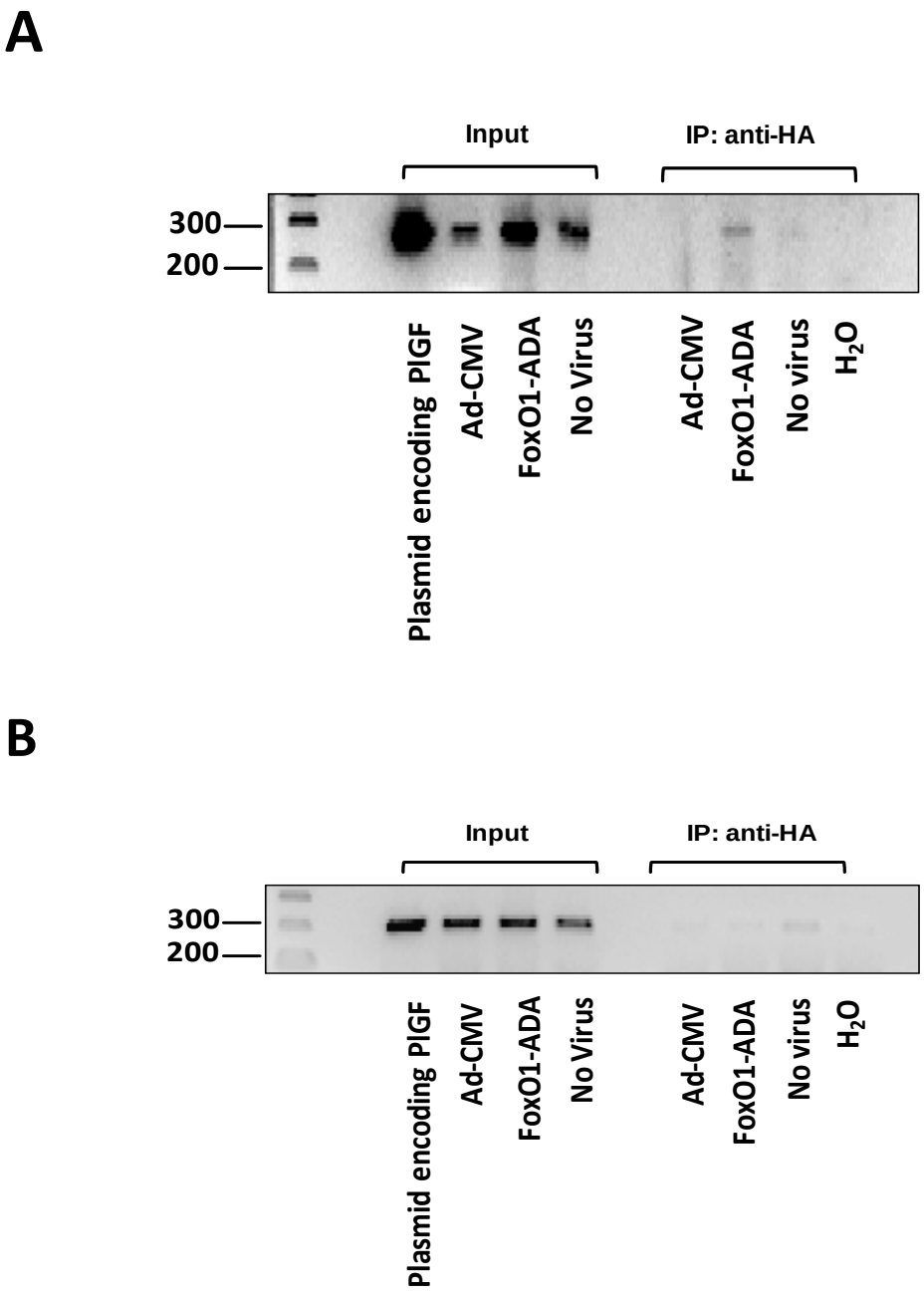


Figure 4.2.9. ***PCR amplification of anti-HA-immunoprecipitated fragments from HUVEC.*** *PCR amplification of DNA fragments from ChIP analysis performed on A, PlGF-seq 1, plasmid encoding PlGF and HUVEC transduced with Ad-CMV, FOXO1-ADA, or non-infected cells.* ***B****, PlGF-seq 2, plasmid encoding PlGF and HUVEC transduced with Ad-CMV, FoxO1-ADA adenoviruses or non-infected. This is a representative gel of 3 independent experiments.*

4.2.11. Ets-2 role in PlGF expression in HUVEC.

FoxO transcription factors have been reported to interact other transcription factors (Randi et al., 2009). The direct interaction of FoxO and ETS transcription factors in the regulation of endothelial cell gene expression and angiogenesis was shown previously (De Val et al., 2008). To examine whether Ets-2 plays a role in PlGF expression in HUVEC, Ets-2 was silenced using siRNA as described in the Methods (Section 2.6.1). After 24 hour incubation in growth factor-free MCDB 131 medium containing 10% FBS, PlGF release was quantified by ELISA. As shown in Figure 4.2.10, the loss of Ets-2 significantly induced PlGF expression compared with control ($p > 0.05$, $n = 3$). This raises the possibility that there might be an interaction between FOXO1 and Ets-2 that results in the suppression of PlGF expression. The role of Ets-2 in the regulation of PlGF expression through interaction with FOXO1 can further be determined by combined knockdown of both transcription factors at the same time and PlGF mRNA and protein quantified to prove whether the interaction of FOXO1 with Ets-2 suppresses that activity of the latter.

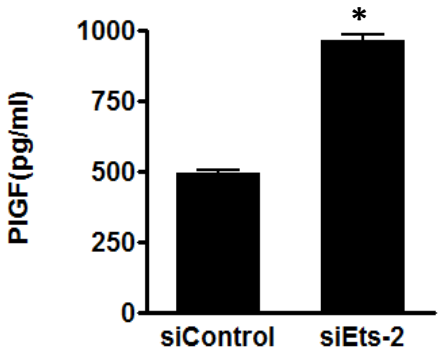


Figure 4.2.10. ***Ets-2 loss-of-function effect on PlGF release in HUVEC.*** *HUVEC were electroporated with siRNA targeted to Ets-2 or control siRNA using an Amaxa nucleofector and incubated overnight. After further 24 hour incubation in growth factor-free MCDB 131 medium containing 10% FBS, PlGF was measured by ELISA in cell-conditioned medium. (*P<0.05, n=3).*

4.2.12. Effect of FOXO1 knockdown on VEGFR-1 expression in HUVEC.

To confirm whether VEGFR-1 expression is regulated by FoxO1 as predicted by reported gene array data (Daly et al., 2004; Potente et al., 2005), HUVEC were electroporated with siRNA oligonucleotides targeted against FOXO1. Cell lysates of FOXO1-koncked down HUVEC were Western blotted using antibodies recognising VEGFR-1 indicated that the ablation of FOXO1 reduced both membrane-bound VEGFR-1 and its soluble form, s-VEGFR-1, at the protein level compared with control. The same cell lysates were blotted with an antibody recognising β-actin to verify equal loading. These data confirm the array data of previous studies and indicate that FOXO1 activity plays a role in the regulation of PlGF expression (Figure 4.2.11).

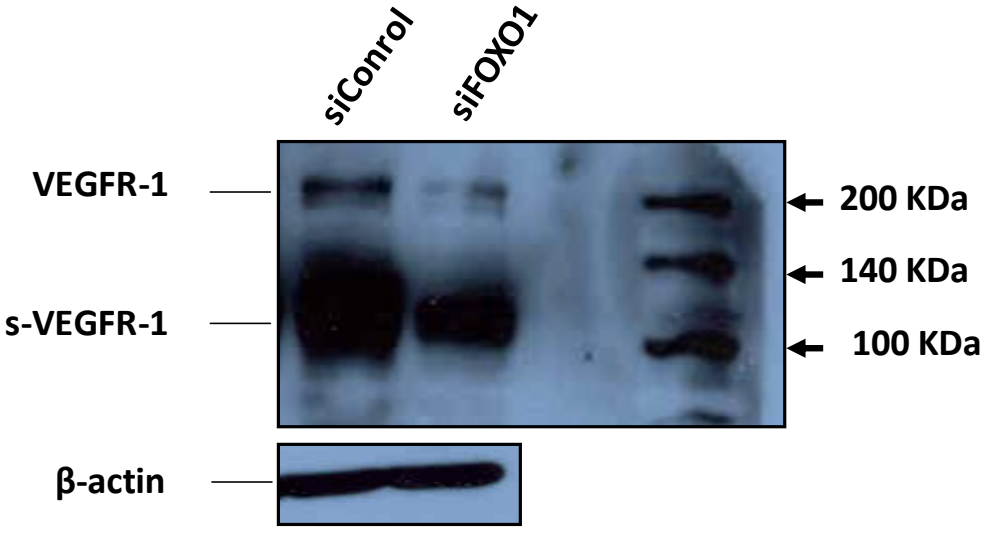


Figure 4.2.11. ***Effect of FOXO1 knockdown on VEGFR-1 expression in HUVEC.*** *Confluent HUVEC were electroporated with siRNA targeted to FOXO1, or control siRNA, using an Amaxa nucleofector and incubated overnight. After 24 hour incubation in growth factor-free MCDB 131 medium, cell lysates were Western blotted using antibodies recognising VEGFR-1 and β-actin. This is a representative blot of 3 independent experiments.*

4.2.13. Effect of FOXO1 gain-of-function on VEGFR-1 expression in HUVEC.

To examine whether FOXO1 gain-of-function would have the opposite effect to FOXO1 knockdown, HUVEC were transduced with FoxO1-ADA, or β-gal adenoviruses overnight followed by 8 hour and qPCR for VEGFR-1 mRNA expression was performed. As can be seen in Figure 4.2.12A, constitutively-active FoxO1 significantly induced VEGFR-1 expression ($p < 0.05$, $n = 3$). FoxO1-ADA over-expression for 24 hours induced VEGFR-1 protein synthesis as demonstrated by Western blotting analysis using an antibody recognising VEGFR-1 in HUVEC lysates. Equal loading was confirmed using antibody against β-actin on the same blots (Figure 4.2.12B). These data confirm the role of FOXO1 in the regulation of VEGFR-1 expression.

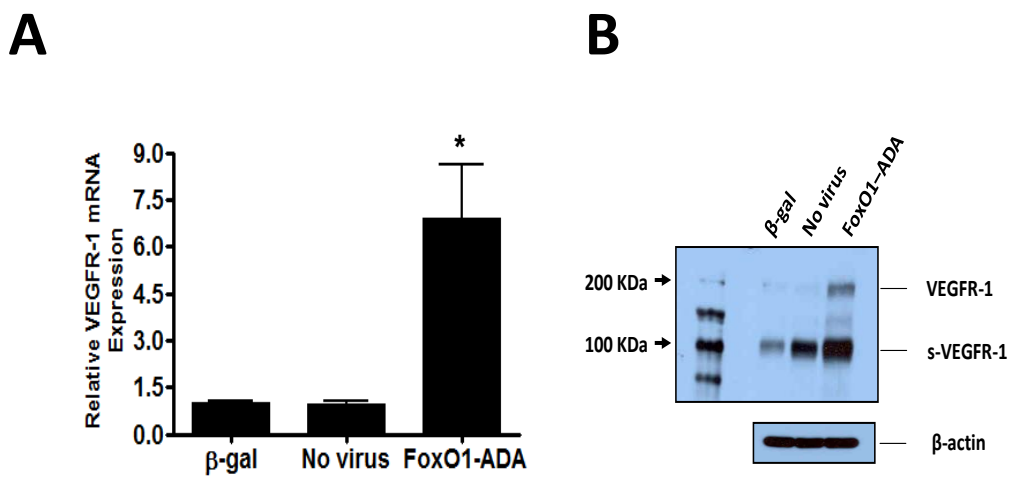
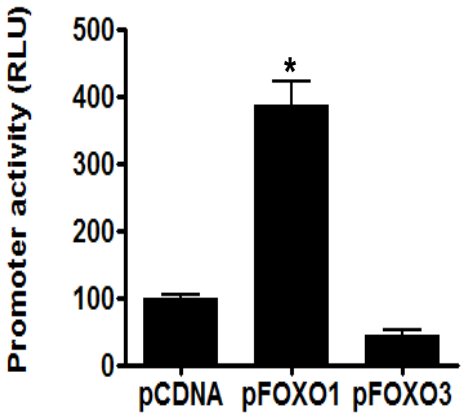


Figure 4.2.12. ***Effect of FoxO1 gain-of-function on VEGFR-1 expression in HUVEC.*** *Confluent HUVEC were transduced with FoxO1-ADA and β-gal adenoviruses overnight followed by 24 hour incubation in growth factor-free MCDB 131 medium containing 10% FBS.* ***A****, VEGFR-1 qPCR was performed.* ***B****, Cell lysates were Western blotted using antibodies recognising VEGFR-1 and β-actin. (*P<0.05, n=3). This is a representative blot of 3 independent experiments.*

4.2.14. Effect of FOXO activity on VEGFR-1 promoter activity in endothelial cells.

As FOXO1 and FOXO3a are the predominant FOXO factors expressed in endothelial cells (Potente et al., 2005). The effects of constitutively-active FOXO1 and FOXO3a on VEGFR-1 promoter activity were examined. Bovine aortic endothelial cells (BAEC) were used for these experiments due to the very low transfection efficiency of plasmids that can be achieved in HUVEC. BAEC were co-transfected with a VEGFR-1 promoter/luciferase reporter plasmid in combination with plasmids expressing constitutively-active FOXO1, FOXO3a or pCDNA control vectors for 24 hours, and VEGFR-1 promoter activity was quantified using a luminometer as described in the Methods (Section 2.6.2). As shown in Figure 4.2.13A, FOXO1 significantly induced VEGFR-1 promoter activity (~ 4-fold) compared with control ($p < 0.05$, $n = 3$), whereas the constitutively-active FOXO3a-expressing plasmid had no effect ($p > 0.05$, $n = 3$). To confirm FOXO1 regulation of VEGFR-1 promoter, BAEC were co-transduced with FoxO1-ADA, or β-gal with VEGFR-1 promoter luciferase reporter plasmids containing 1.3 Kb or 0.9 Kb (Both constructs contain a FoxO1 binding site, see details below) of the VEGFR-1 promoter sequence upstream of transcription site, or the empty pGL2 vector for 24 hours. As shown in Figure 4.2.13B, constitutively-active FOXO1 increased the activity of the (1.3 Kb construct) by almost 15-fold and the 0.9 Kb construct by over 30-fold compared to β-gal control ($p < 0.01$, $n = 3$), but had no effect on pGL2 control vector activity. These data confirm FOXO1 interaction/regulation of the VEGFR-1 promoter. The discrepancy in the observed VEGFR-1 promoter activity between the two constructs suggests that the long construct might contain a transcription repressor in addition to a FOXO1 binding site.

A



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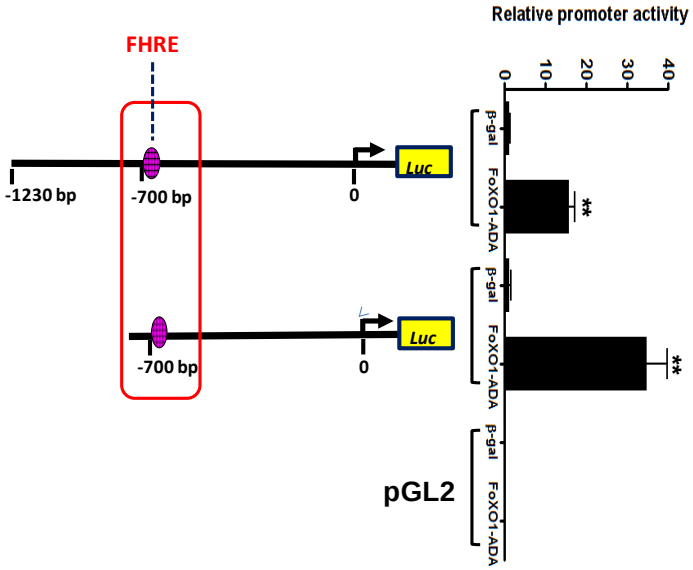

Figure 4.2.13*. **Effect of FoxO1 gain-of-function effect on VEGFR-1 promoter activity in endothelial cells.** VEGFR-1 promoter activity was quantified using the luciferase reporter assay. BAEC were co-transduced with **A**, VEGFR-1 promoter reporter plasmid with constitutively-active FoxO1, constitutively-active FoxO3a and pCDNA control vectors for 24 hours. **B**, VEGFR-1 promoter reporter plasmid with either FoxO1-ADA adenovirus or pGL2 Plasmid were transduced in BAEC with FoxO1-ADA, or β-gal. (*P<0.05, **P<0.01, n=3). 0, transcription start site. FHRE, forkhead recognition elements. LUC, luciferase reporter gene.*

4.2.14. Does FOXO1 directly interact with the VEGFR-1 promoter?

To identify potential FOXO1 binding sites on the VEGFR-1 gene promoter, conserved regions in the 5' region of the VEGFR-1 gene were identified by alignment of 10 Kb sequences immediately upstream the transcription start site from human, chimpanzee, marmoset, pig, cow, rat and mouse using the aforementioned search engines. One conserved FoxO binding site that lies between -761 to -751 bp was identified (Figure 4.2.14A) upstream of the transcriptional start site and was present in both VEGFR-1 promoter constructs, as indicated on Figure 4.2.15B.

To visually demonstrate whether FOXO1 directly binds to the VEGFR-1 gene promoter, ChIP analysis was again performed on HUVEC transduced with FoxO1-ADA, Ad-CMV adenoviruses, or non-infected cells for 48 hours. Chromatin-bound DNA was immunoprecipitated with an antibody against the HA epitope (HA-tagged FOXO1-ADA). Immunoprecipitated DNA was analysed by PCR using primers flanking a region of ~ 262 bp in size, which lies between -882 to - 619 bp upstream the transcription start site and contains the conserved putative FOXO1 binding site on the VEGFR-1 promoter (4.2.14A). PCR amplification of the immunoprecipitated DNA fragments (specific Band) from cells transduced with FoxO1-ADA, but not from Ad-CMV-transduced, or non-infected HUVEC indicates that FOXO1 directly interacts with the VEGFR-1 promoter at this site (Figure 4.2.14B).

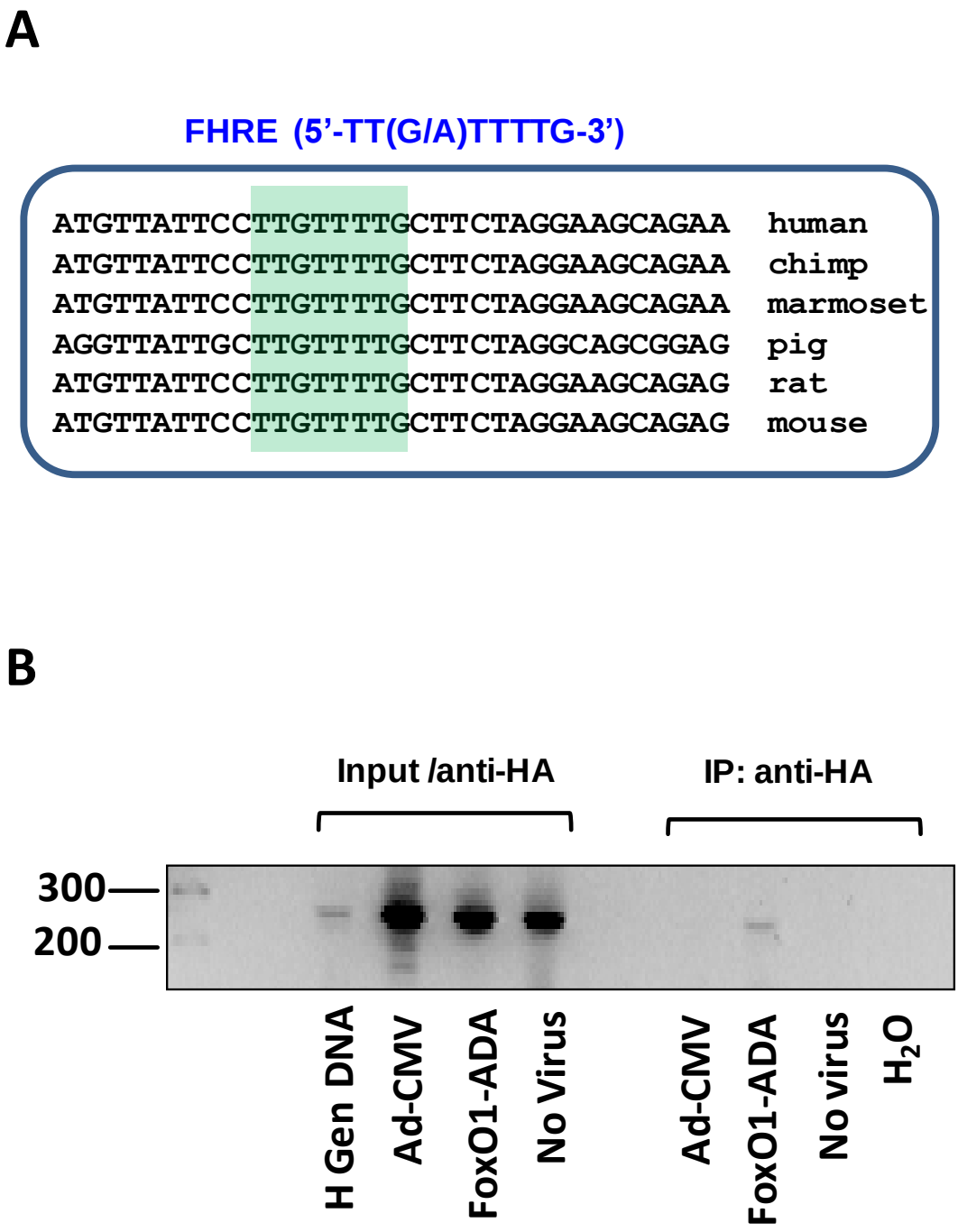

Figure 4.2.14. ***ChIP on FoxO1 binding site on the VEGFR-1 gene promoter in HUVEC. A,*** *identified putative FoxO1 binding site in the VEGFR-1 promoter in a conserved region across several species.* ***B,*** *PCR amplification of anti-HA-immunoprecipitated fragments from HUVEC transduced with Ad-CMV, FoxO1-ADA adenoviruses, and non-infected cells and human genomic DNA control. This is a representative gel of 3 independent experiments.*

4.3. Discussion

In this study, direct evidence demonstrating a positive role for FOXO1 in the regulation of PlGF and its receptor, VEGFR-1, expression in human endothelial cells is presented for the first time. Dysregulation of FoxO1 activity by hyperglycaemia in insulin-responsive tissues is a hallmark of metabolic disorders. In atherosclerosis, the loss of endothelial integrity, increased oxidative stress, activation of pro-inflammatory pathways and the up-regulation of PlGF and its receptor VEGFR-1 have all been documented (Autiero et al., 2003a; Carmeliet et al., 2001; Luttun et al., 2002).

The data from Chapter 3 indicated that inhibition of the PI3K/Akt signalling pathway significantly augmented PlGF levels in cultured endothelial cells *in vitro*, and in the liver and plasma of obese *ob/ob* mice. NF-κB is a transcription factor that has been widely implicated in inflammation (Baker et al., 2011), and putative binding sites for NF-κB on the PlGF gene promoter have been reported (Cramer et al., 2005). In endothelial cells, NF-κB mediates the cytotoxic effects of TNF-α through the activation of the PI3K/Akt pathway (Zhou et al., 2008). These findings provided a rationale to initially examine whether NF-κB is implicated in hyperglycaemia-mediated regulation of PlGF expression in endothelial cells. However, the inhibition of NF-κB transcription activity using the pharmacological inhibitor, isohelenin, or the dominant-negative IKK adenovirus was found to have no effect on PlGF expression in HUVEC. Others have shown NF-κB activation in HUVEC after a short period of hyperglycaemia (Morigi et al., 1998). We have not repeated these experiments, so our findings should be taken with some caution.

The dual role of FOXO1 in the regulation of angiogenesis and metabolism associated with the suppression of PI3K/Akt and PlGF up-regulation reported in *ob/ob* mouse

model (Voros et al., 2005) warranted the examination of the role of this transcription factor in the regulation of PlGF expression in endothelial cells. In the previous Chapter, it was shown that hyperglycaemia-mediated inhibition of Akt activity results in the suppression of FOXO1 phosphorylation in cultured HUVEC and that this effect can be reversed by stimulation with IGF-1. In insulin–responsive tissues, one of the major effects of persistent FOXO1 nuclear localisation, resulting from chronic hyperglycaemia and blunting of insulin signalling are metabolic abnormalities culminating in insulin resistance and induction of pro-inflammatory gene expression; two key triggers in the development of atherosclerosis. In endothelial cells, Tanaka et al., (2009) found that incubation in 25 mM D-glucose or 500 μM H_2O_2, both significantly induced FOXO1 promoter activity compared with control. Using both gain- and loss-of-function approaches, in this study it was shown that the loss of FOXO1 activity in HUVEC cultured under normal conditions significantly suppressed PlGF release compared with control. Furthermore, FOXO1 ablation abolished the induction of PlGF expression in hyperglycaemia. Conversely, adenovirus-mediated over-expression of nuclear constitutively–active FoxO1 significantly induced PlGF release in both the absence and presence of IGF-1, demonstrating that FOXO1 is the major downstream effector regulating PlGF expression in this pathway. To verify that FOXO1 regulates PlGF expression *in vivo*, FVB/N mice were transduced with FoxO1-ADA adenovirus over 48 hours leading to elevated PlGF mRNA in the liver and circulating PlGF protein in plasma compared with Ad-CMV-transduced control mice. Taken together, the data identify FOXO1 as a transcription regulator of PlGF.

FoxO factors are known to regulate gene transcription by either directly binding the DNA of target genes, or indirectly in conjunction with other transcription factors through protein-protein interactions. Performing ChIP followed by PCR amplification

of evolutionary conserved FOXO1 binding sites on PlGF promoter in HUVEC clearly demonstrated that FOXO1 regulation of PlGF is through direct binding. In another study, PlGF expression was up-regulated by FoxD1/BF-2, which is exclusively expressed by stromal cells in the embryonic renal cortex during development. FoxD1/BF-2 binds PlGF promoter at a HNF-3β site (Zhang et al., 2003). Similarly, the forkhead-related factor, glial cell missing-1(GCM-1) is reported to regulate PlGF transcription in placental trophoblasts. The over-expression of GCM-1 was reported to prevent the inhibitory effects of hypoxia on PlGF expression (Chang et al., 2008). Moreover, the knockdown of Ets-2, a repressor factor and a member of the ETS family which have been shown to interact with FoxO factors in the regulation of angiogenesis (De Val et al., 2008) resulted in a pronounced increase in PlGF release. The role of ETS factors in the regulation of PlGF remains to be fully elucidated.

Altered VEGFR-1 expression following FoxO1-ADA over-expression in endothelial cells was reported in gene arrays of two independent studies (Daly et al., 2004; Potente et al., 2005). In these studies using gain- and loss-of-function approaches was used to demonstrate how FOXO1 regulates VEGFR-1 expression and identify potential binding sites for FOXO1 on the VEGFR-1 promoter. Silencing FOXO1 with siRNA in HUVEC significantly reduced the expression of both membrane-bound and soluble forms of VEGFR-1 compared with control. Conversely, HUVEC transfected with the FoxO1-ADA that is permanently retained in the nucleus significantly increased the expression of VEGFR-1 at the protein level compared with β-gal and non-infected cells. This was confirmed by qPCR on samples from the same experiments. This finding does not however rule out the possibility of indirect interaction under different circumstances. To verify whether there is an overlap between FOXO factors in the regulation of VEGFR-1, BAEC were co-transfected with plasmids over-expressing

either constitutively-active FoxO1 or FoxO3a with VEGFR-1 promoter plasmid reporter. These experiments showed that only FoxO1 induced VEGFR-1 promoter activity and that FoxO3a and pCDNA control were without effect. This was further corroborated by co-transfection of FoxO1-ADA adenovirus with VEGFR-1-expressing reporter plasmid. Performing ChIP on HUVEC transfected with FoxO-1-ADA and Ad-CMV indicated that FOXO1-ADA binds to the (TT(G/A)TTTTG) binding site on the VEGFR-1 promoter.

The discovery that FOXO1 activation promotes PlGF and VEGFR-1 expression in endothelial cells may provide an explanation for the shift towards pro-inflammatory signalling in angiogenic pathologies. It is also possible to speculate that the promotion of PlGF and VEGFR-1 expression which was previously shown to promote endothelial cells survival via VEGFR-1 and activation of the PI3K/Akt signalling pathway (Cai et al., 2003b) may to contribute to the angiogenic switch to compensate for the loss of Akt activity in endothelial dysfunction. Furthermore, the up-regulation PlGF and VEGFR-1 in pathology might have implications on the crosstalk at the ligand level between PlGF and VEGF and the receptor level between VEGFR-1 and VEGFR-2 in the regulation of angiogenesis. These data also add to the body of evidence placing FOXO factors, as actors, at the centre of two interlinked loops, metabolic and angiogenic, each of which feeds the other and compound its deleterious effects.

Taken together, these data implicate FOXO1 in the direct regulation of PlGF and VEGFR-1 expression. FOXO1 controlled and tissue-specific expression may therefore provide an excellent target for therapy in pathologies characterised by inflammation and endothelial dysfunction.

Chapter 5

Mechanisms mediating VEGF up-regulation of PlGF release in endothelial cells

5.1. Introduction

VEGF elicits its major biological effects, such as endothelial cell migration, survival, proliferation and tube formation via binding to high affinity VEGFR-1 and VEGFR-2 kinase receptors (de Vries et al., 1992; Terman et al., 1992b). PI3K/Akt, MAPK and PKC are major intracellular signalling pathways downstream of VEGFR-1 and VEGFR-2, and their activation by VEGF and other growth factors modulates the expression of a wide range of genes in different cell types (Ferrara et al., 2003). In endothelial cells, VEGF enhances angiogenesis by augmentation of NO production and the promotion of endothelial cell survival (Dvorak et al., 1995; Fujio and Walsh, 1999). VEGF can also enhance angiogenesis through the induction of other pro-angiogenic genes, including PlGF (Carmeliet et al., 2001; Yao et al., 2005). In recent years considerable work has been undertaken to define the mechanisms through which PlGF contributes to pathology, yet little is known about its regulation and release. As outlined in the Introduction, there are several mechanisms by which PlGF can enhance VEGF activity, including displacement of VEGF from VEGFR-1, making it freely available to bind VEGFR-2, or the preferential formation of PlGF:VEGF heterodimers, thus reducing the formation of the more potent VEGF:VEGF homodimers (Cao et al., 1996; Eriksson et al., 2002).

In Chapter 4 of this thesis, it was demonstrated that the inhibition of the PI3K/Akt signalling pathway in endothelial cells enhanced PlGF release. Constitutive activation of Akt or stimulation of Akt activity with IGF-1 suppressed PlGF expression through the inactivation of the forkhead transcription factor, FOXO1. VEGF also promotes PlGF expression despite strongly activating Akt (Yao et al., 2005; Zhao et al., 2004).

This puzzling paradox prompted the attempt to define the mechanism of VEGF-mediated induction of PlGF expression in endothelial cells and whether the endogenous VEGF plays a significant role in this process.

5.2. Results

5.2.1. Effect of exogenous VEGF stimulation on PlGF release in endothelial cells.

To explore the role of VEGF in the regulation of PlGF release in endothelial cells, HUVEC were made quiescent by growth factor starvation for 6-8 hours in growth factor-free MCDB 131 medium containing 10% FBS. Following 24 hour incubation in fresh MCDB 131 medium in the presence of increasing concentrations (10 and 20 ng/ml) of recombinant $VEGF_{165}$ and PlGF was quantified in the culture medium by ELISA. As shown in Figure 5.2.1A, $VEGF_{165}$ (20 ng/ml) significantly increased PlGF secretion compared with non-treated cells ($p < 0.05$, $n = 3$). Similarly, HAEC were stimulated with 20 ng/ml of recombinant $VEGF_{165}$ for 24 hours showed a pronounced increase with VEGF stimulation (approximately 6-fold) in PlGF release compared with control ($p < 0.05$, $n = 3$, Figure 5.2.1B), confirming findings of other published reports (Yao et al., 2005; Zhao et al., 2004).

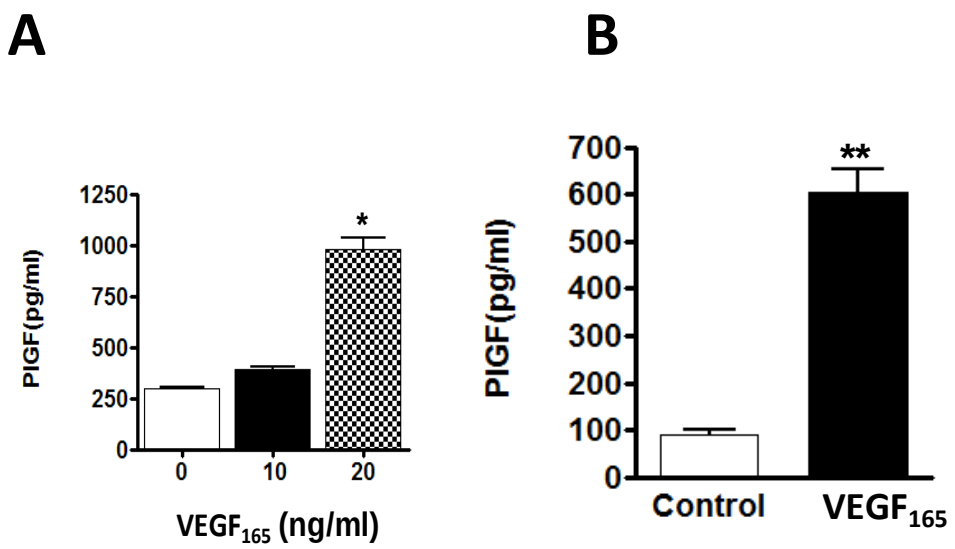

Figure 5.2.1. ***Effect of exogenous VEGF165 on PlGF release in endothelial cells.*** ***A****, Confluent HUVEC were incubated in growth factor-free MCDB 131 medium containing 10% FBS. Cells were then stimulated with increasing concentrations of $VEGF_{165}$ for 24 hours in fresh medium.* ***B****, Confluent HAEC were incubated in growth factor-free MCDB 131 medium containing 10% FBS. Cells were then stimulated with 20 ng/ml of $VEGF_{165}$, or control and incubated for 24 hours in fresh MCDB 131 medium containing 10% FBS. PlGF was quantified in cell-conditioned medium by ELISA. (*P<0.05, n=3).*

5.2.2. Effect of endogenous $VEGF_{164}$ stimulation on PlGF release in HUVEC.

To confirm exogenous $VEGF_{165}$ effect on PlGF release, over-expression of murine $VEGF_{164}$ (in mouse; the human proteins are one residue longer), or β-gal in HUVEC was achieved by recombinant adenoviruses as described in the Methods (Section 2.5). As depicted in Figure 5.2.2, the over-expression of VEGF significantly induced PlGF release in HUVEC compared with control ($p < 0.01$, $n = 3$). These data indicate that the addition of recombinant VEGF or adenovirus-mediated $VEGF_{164}$ over-expression stimulate PlGF secretion in endothelial cells.

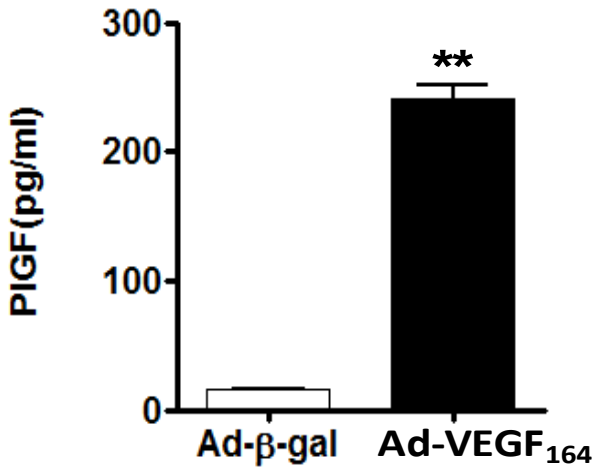

Figure 5.2.2. ***Effect of VEGF164 on PlGF release in HUVEC.*** *HUVEC transduced with adenoviruses encoding murine $VEGF_{164}$ or β-gal overnight, followed by 24 hour incubation in growth factor-free MCDB 131 medium containing 10% FBS. PlGF was quantified in cel supernatants by ELISA. (**P<0.01, n=3).*

5.2.3. The role of VEGFR-2 in VEGF-mediated PlGF release in HUVEC.

It has previously been documented that VEGF-mediates the induction of PlGF secretion in bovine microvascular endothelial cells through the use of neutralising antibodies to prevent VEGF interacting with its receptors (Zhao et al., 2004). To confirm this finding, an alternative approach was adopted using an siRNA duplex to knockdown VEGFR-2 synthesis in HUVEC in the presence of VEGF (20 ng/ml). VEGFR-2 knockdown in HUVEC cell lysates was verified using a VEGFR-2 ELISA employing a monoclonal anti-VEGFR-2 as a capture antibody, and biotinylated anti-VEGFR-2 as a detection antibody. This VEGFR-2 ELISA was developed in-house and validated by Dr Melissa Cudmore. Serial dilutions of the recombinant VEGFR-2 were used as standard curve for ELISA (Figure 5.2.3A). As can be seen in Figure 5.2.3B, VEGFR-2

protein synthesis was almost completely abolished by siRNA compared with control ($p < 0.01$, $n = 3$).

The data shown in Figure 5.2.3C, indicate that VEGFR-2 knockdown alone in the absence of VEGF reduced PlGF secreted levels compared with control, although it did not reach statistical significance ($p > 0.05$, $n = 3$). However, induction of PlGF secretion by VEGF was significantly inhibited in the absence of VEGFR-2 ($p < 0.05$, $n = 3$). In order to further define the role VEGFR-2, HUVEC were treated with the VEGFR-2 activation inhibitor, SU5416 (20 μM) followed by 24 hour incubation in the presence of VEGF (20 ng/ml), or control. As shown in Figure 5.2.3D, VEGF alone significantly induced PlGF release compared with control. This induction was, however dramatically reduced in the presence of SU5416. These data implicate VEGFR-2 in VEGF-mediated induction of PlGF release in endothelial cells.

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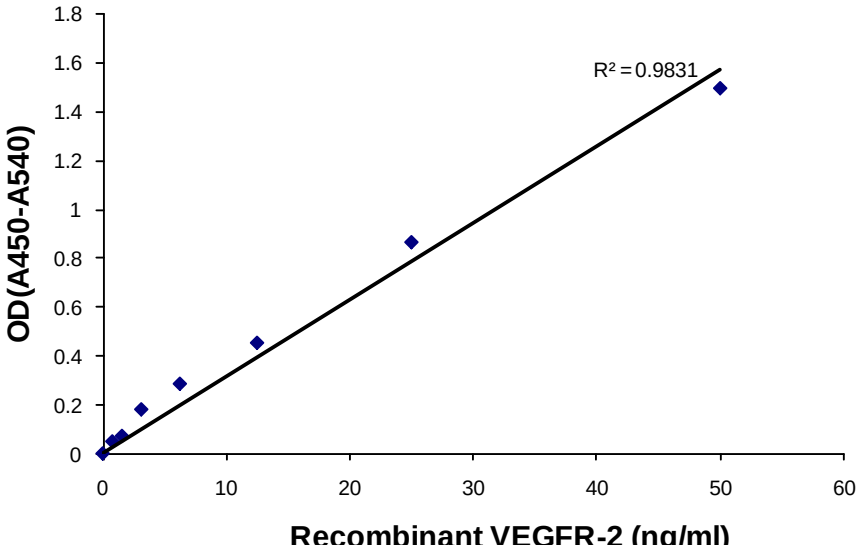

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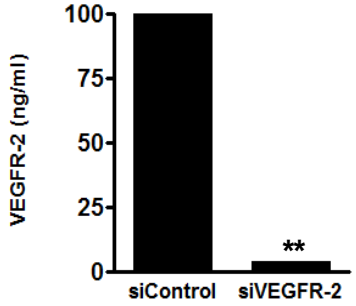

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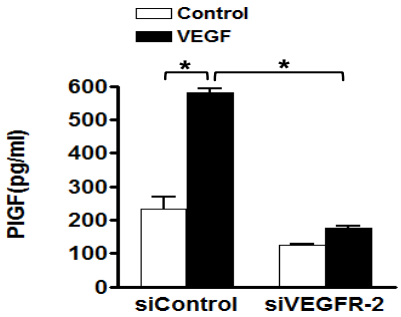

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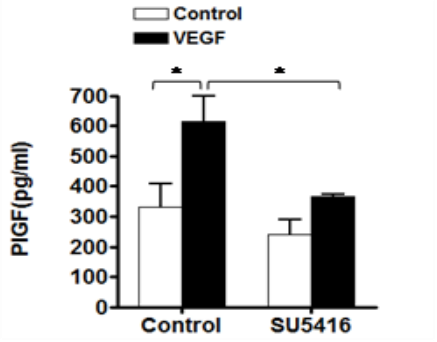

Figure 5.2.3. ***The role of VEGFR-2 in the regulation of VEGF-induced PlGF release in HUVEC****. HUVEC were electroporated with siRNA targeted to VEGFR-2 or control siRNA using an Amaxa nucleofector and incubated overnight.* ***A****,a typical VEGFR-2 ELISA standard curve (see the Methods section).* ***B****, After 24 hour incubation, VEGR-2 was quantified in cell lysates by VEGFR-2 ELISA.* ***C****, After 24 hour incubation in growth factor-free MCDB 131 medium containing 10% FBS, and VEGFR-2 levels were quantified by ELISA in cell-conditioned medium.* ***D,*** *HUVEC were treated with SU5416 (20 μM) or vehicle and incubated in growth factor-free MCDB 131 medium containing 10% FBS in the presence of VEGF (20ng/ml) or control. After 24 hours, PlGF was measured by ELISA in cell-conditioned medium. (*P<0.05, **P<0.01, n=3).*

5.2.4. The role of VEGFR-1 in VEGF-mediated PlGF release in HUVEC

It has been reported that VEGFR-1 does not play a role in VEGF stimulation of VEGF secretion in endothelial cells (Zhao et al., 2004). To explore this finding further, an siRNA duplex targeted to block VEGFR-1 expression was introduced into HUVEC and the PlGF levels secreted were quantified by ELISA in the cell-conditioned medium. As depicted in Figure 5.2.4A, the loss of VEGFR-1 strongly induced PlGF release compared with control ($p < 0.05$, $n = 3$), suggesting a role for VEGFR-1 in the regulation of PlGF expression.

To further explore the role of VEGFR-1 in the regulation of VEGF-mediated up-regulation of PlGF release, VEGFR-1 was silenced in HUVEC in the presence or absence of adenoviruses over-expressing murine PlGF-2, $VEGF_{164}$, or β-gal control. The data are shown in Figure 5.2.4B. The loss of VEGFR-1 markedly induced PlGF release under all conditions compared with control siRNA-treated cells (Ad-$VEGF_{164}$, $P < 0.05$, β-gal, $P < 0.01$, $n = 3$), indicating that, unlike VEGFR-2, VEGFR-1 may actually play an inhibitory role in mediating PlGF release. However, in control siRNA-treated cells, murine PlGF over-expression actually seemed to increase PlGF secretion. This is surprising, if VEGFR-1 is negative regulator of PlGF expression, a possible explanation is that high levels of secreted murine PlGF-2 are cross-reacting with the human ELISA.

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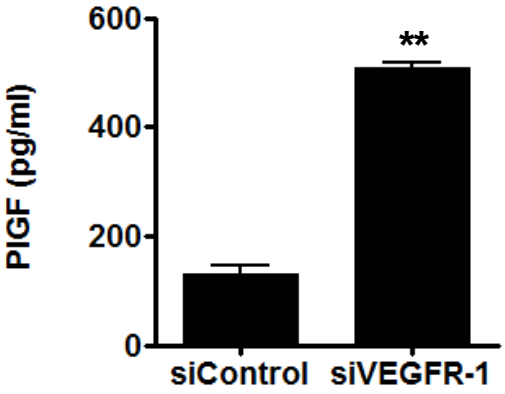

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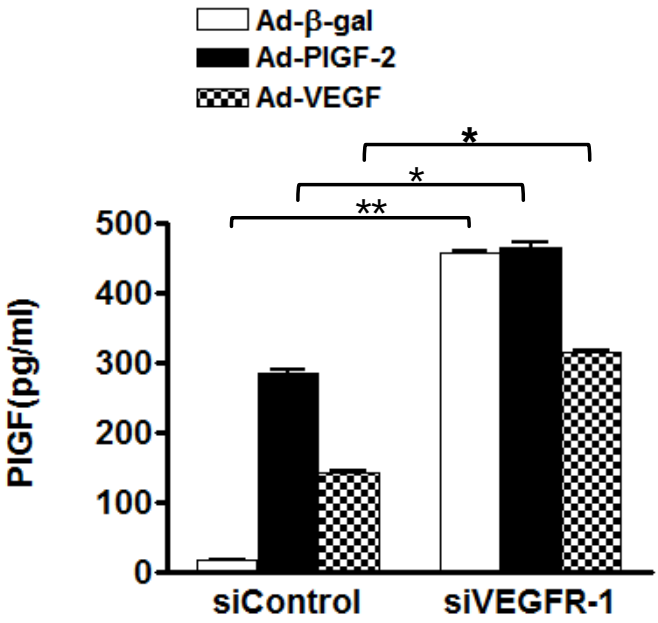


Figure 5.2.4. ***The role of VEGFR-1 in the regulation of VEGF-induced PlGF release in HUVEC.*** *HUVEC were electroporated with siRNA targeted to VEGFR-1 or control siRNA using an Amaxa nucleofector and incubated overnight.* ***A****, After 24 hour incubation in growth factor-free MCDB 131 medium containing 10 FBS%, PlGF was measured by ELISA in cell-conditioned medium.* ***B,*** *cells were then transduced with adenoviruses encoding murine PlGF-2, $VEGF_{164}$ or β-gal, and incubated overnight. After 24 hour incubation in growth factor-free MCDB 131 medium containing 10% FBS, PlGF was measured by ELISA in cell-conditioned medium. (*P<0.05, **P<0.01. n=3).*

5.2.5. Effect of VEGF knockdown on PlGF release in HUVEC.

Since stimulation of endothelial cells with exogenous $VEGF_{165}$, or over-expression of $VEGF_{164}$ was found to induce PlGF release, this raised the question whether the knockdown of endogenous VEGF would have the converse effect. Zhao et al., (2004) and Yao et al., (2005) showed previously that antibodies to VEGF could inhibit PlGF release. The effect of VEGF knockdown was examined by silencing VEGF in HUVEC by siRNA. As shown in the Figure 5.2.5. Interestingly, VEGF ablation significantly increased the release of PlGF compared with control ($p < 0.05$, $n = 4$). It is Conceivable that the observed increase in PlGF release is due to a loss of PlGF:VEGF heterodimer formation , which should preferentially form within the cell upon the loss of VEGF. Alternatively, the loss of VEGF may invoke other mechanisms to compensate for its own loss to maintain homeostasis.

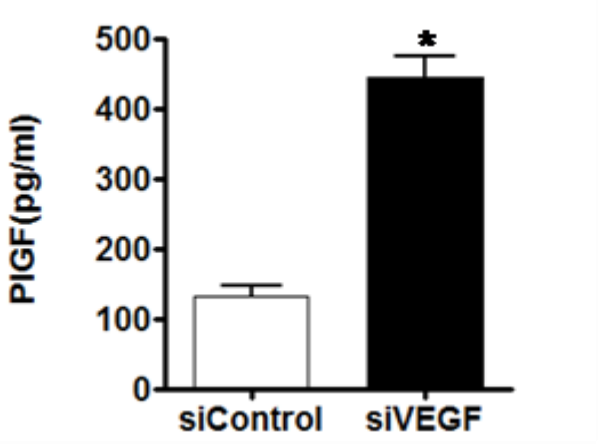
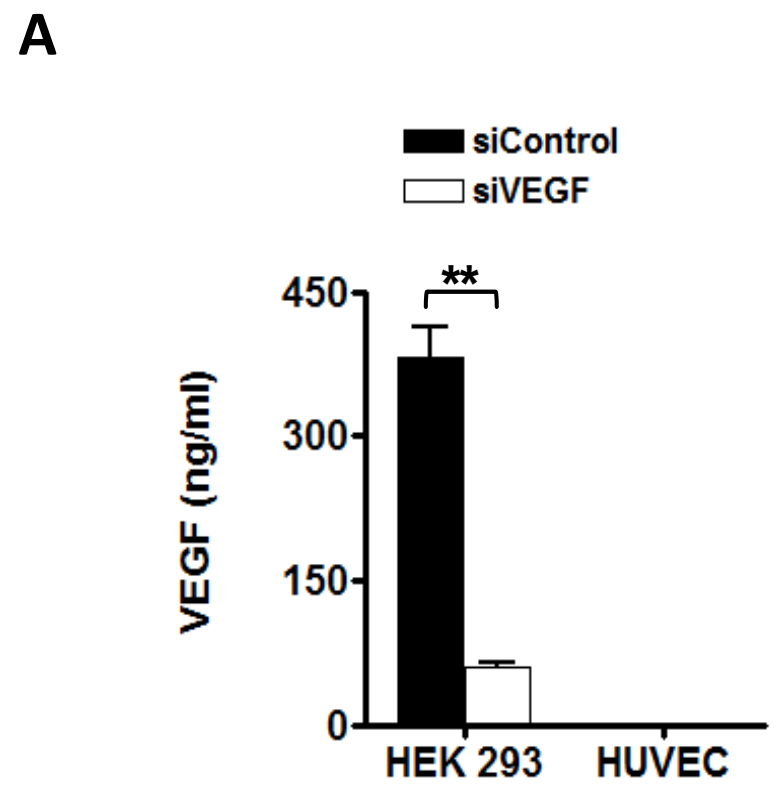


Figure 5.2.5. ***Effect of VEGF knockdown on PlGF release in HUVEC.*** *HUVEC were electroporated with siRNA targeted to VEGF, or control siRNA using an Amaxa nucleofector and incubated overnight. After 24 hour incubation in growth factor-free MCDB 131 medium containing 10% FBS, PlGF was measured by ELISA in cell-conditioned medium. (*P<0.05, n=4).*

5.2.6. Detection of endogenous VEGF in HUVEC

The puzzling and seemingly contradictory finding that both VEGF stimulation with and knockdown of endogenous VEGF induced PlGF expression warranted the examination of VEGF knockdown further using HEK 293 cells as a positive control. Following the electroporation of HEK 293 cells with VEGF siRNA, VEGF mRNA expression was reduced by ~ 50% and the secretion of VEGF protein by > 70% compared with siRNA control (mRNA, $p < 0.05$, protein, $p< 0.01$, $n = 3$, Figure 5.2.6 A and B). In HUVEC, however, VEGF protein was not detectable using the same ELISA (Figure 5.2.6A). in addition, very low levels of VEGF mRNA were detectable by PCR which appeared to be below the limit of detection for the assay (Figure 5.2.6C). This is supported by the finding that transfection of HUVEC with VEGF siRNA did not result in a reduction in VEGF mRNA in these cells.



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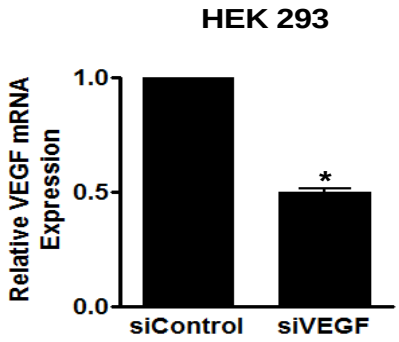

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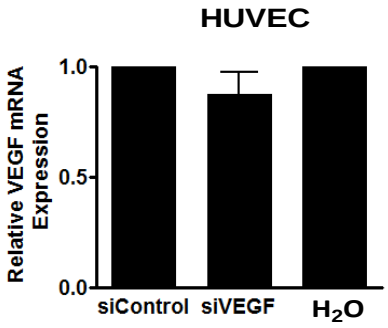


Figure 5.2.6. ***Detection of endogenous VEGF in HUVEC.*** ***A****, HEK 293 and HUVEC were electroporated with siRNA targeted to VEGF, or control siRNA using an Amaxa nucleofector and incubated overnight.* ***A****, after 24 hour incubation, VEGF protein was quantified in cell-conditioned medium by ELISA.* ***B & C****, After an 8 hour incubation in growth factor-free MCDB 131 medium containing 10% FBS, cell lysates were collected, RNA extracted and VEGF qPCR performed. (*P<0.05, **P<0.01, n=3).*

5.2.7. Effect of VEGF induction by hypoxia on PlGF release in HUVEC

Since we were unable to detect VEGF under normoxia, HUVEC were incubated in hypoxia conditions in an attempt to induce VEGF expression (Liu et al., 1995; Wang and Semenza, 1995) and examine its subsequent effect on PlGF release. To this end, VEGF siRNAs were introduced into HUVEC by electroproration and following a 24 hour incubation in normoxia (21% O_2), or hypoxia (1% O_2) in growth factor-free

MCDB 131 medium in the presence of VEGF (20 ng/ml). PlGF was quantified by ELISA in cell supernatants. Consistent with previous findings, both VEGF stimulation and knockdown significantly elevated PlGF release in HUVEC ($p < 0.05$, $n = 3$), whereas there was no significant difference observed in the secreted PlGF levels between normoxia and hypoxia ($p > 0.05$, $n = 3$), indicating that hypoxia alone had no effect on PlGF release. Also, there was no effect of hypoxia on VEGF-mediated induction of PlGF expression by hypoxia (Figure 5.2.7), suggesting, as expected, that VEGF induction of PlGF release in endothelial cells is independent of hypoxia. This finding suggests that the increase in PlGF secretion observed in hypoxia is independent of the up-regulation of endogenous VEGF, but more experiments are needed to prove this finding.

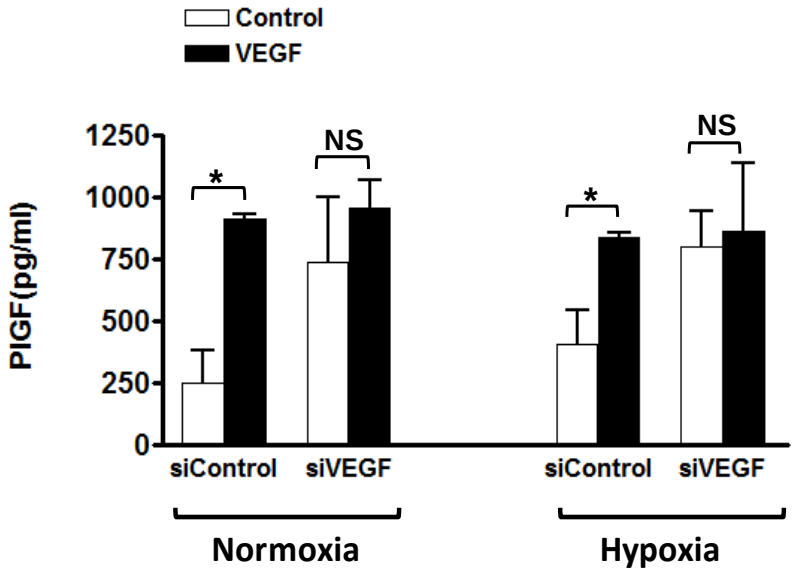
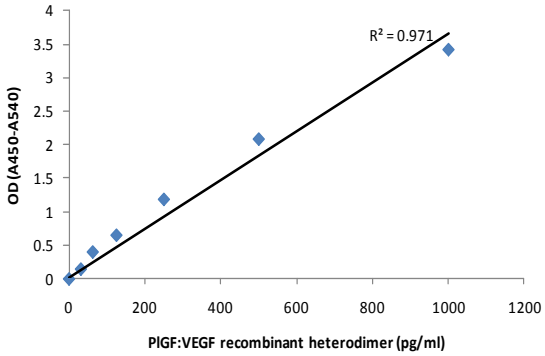


Figure 5.2.7. ***Effect of endogenous VEGF and hypoxia on PlGF release in HUVEC.*** *HUVEC were electroporated with siRNA targeted to VEGF, or control siRNA, using an Amaxa nucleofector overnight. After 24 hour incubation in MCDB 131 medium containing 10% FBS under 1%, or 21 O_2 tension in the presence of VEGF (20 ng/ml), PlGF was measured by ELISA in cell-conditioned medium. (*P<0.05, n=3) .NS = not significant.*

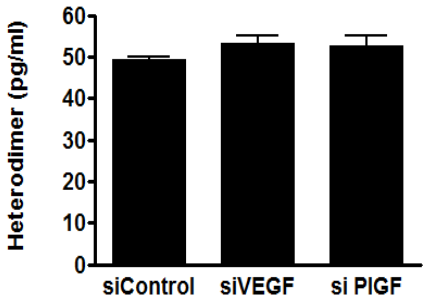
5.2.8. Examination of the putative role of preferential PlGF:VEGF heterodimer formation in the modulation of PlGF release in HUVEC.

The preferential formation of the PlGF:VEGF heterodimers has previously been reported (Cao et al., 1996; DiSalvo et al., 1995) and might play a role in the apparent increase in PlGF secretion observed following VEGF knockdown in HUVEC. Due to our inability to detect VEGF in HUVEC, it was hypothesised that any VEGF protein produced by endothelial cells might present as PlGF:VEGF heterodimers in the presence of higher levels of PlGF. Therefore, it was postulated that VEGF knockdown in HUVEC may allow the formation of more PlGF homodimers, and thus the apparent up-regulation of PlGF detected by ELISA. To test this hypothesis, a PlGF:VEGF heterodimer sandwich ELISA was devised consisting of anti-monoclonal PlGF:VEGF heterodimer monoclonal antibody, and polyclonal anti-VEGF detection antibody. The assay was optimised using recombinant PlGF:VEGF heterodimer standard shown in the Figure 5.2.8 A. Figure 5.2.8B shows that silencing of PlGF or VEGF in HUVEC for 24 hours to allow the formation of PlGF, or VEGF homodimers, did not result in a change in PlGF:VEGF heterodimer levels ($p > 0.05$, $n = 3$), suggesting that VEGF regulation of PlGF release in endothelial cells is PlGF:VEGF heterodimer-independent. PlGF knockdown (4-fold) was verified by PlGF ELISA as shown in Figure 5.2.8C ($p < 0.05$, $n = 3$).

A



B



C

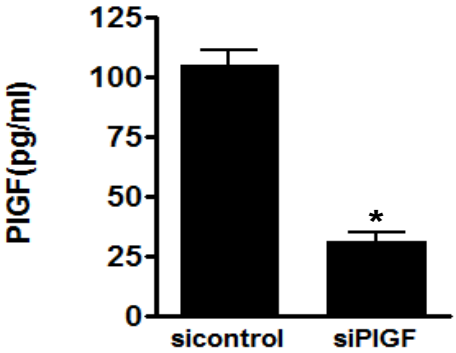


Figure 5.2.8. ***Effect of PlGF:VEGF heterodimer formation on VEGF-mediated PlGF release in HUVEC.*** ***A,*** *PlGF:VEGF heterodimer ELISA standard.* ***B,*** *HUVEC were electroporated with siRNA targeted to VEGF, PlGF or control siRNA using an Amaxa nucleofector overnight. After 24 hour incubation in growth factor-free MCDB 131 medium containing 10% FBS, PlGF:VEGF heterodimer quantified using heterodimer ELISA (see methods).* ***C****, Demonstration of PlGF knockdown in HUVEC. (*P<0.05, n=3).*

5.2.9. The role of PI3K in VEGF induction of PlGF release in HUVEC.

As PI3K is an important mediator of the biological effects of VEGF in endothelial cells, and shown in Section 3.2.6 in Chapter 3 to negatively regulate PlGF expression. The role of PI3K in VEGF-mediated regulation of PlGF release was examined. HUVEC were treated with LY294002 (20 μM) in the presence of VEGF (20 ng/ml), or control as described in Section 3.2.6. As shown in Figure 5.2.9, HUVEC treatment with LY294002 had no effect on VEGF-mediated induction of PlGF release compared with control, suggesting that PI3K is not important for VEGF-mediated regulation of PlGF expression.

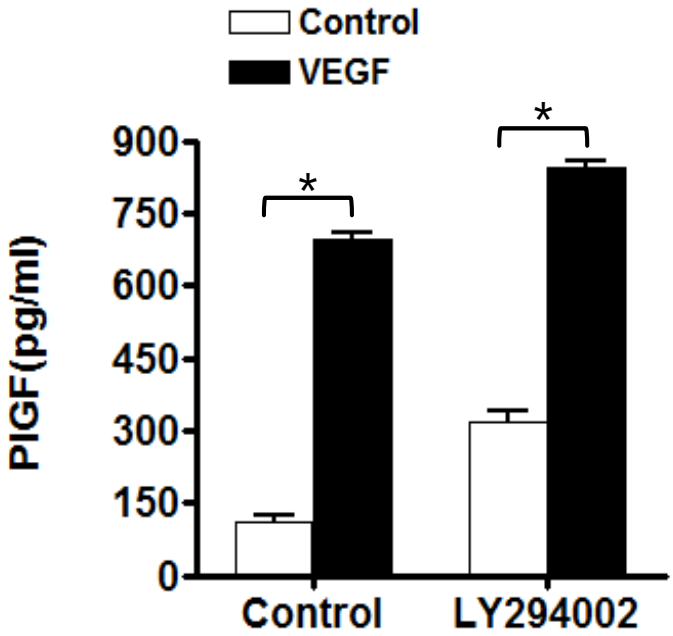


Figure 5.2.9. ***The role of PI3K in VEGF induction of PlGF release in HUVEC.*** *Confluent HUVEC were treated with 20 μM of LY294002, or control. After 24 hour incubation in growth factor-free MCDB 131 medium containing 10% FBS in the presence of VEGF (20ng/ml), or control, PlGF was measured by ELISA in cell-conditioned medium. (*P<0.05, n=3).*

5.2.10. The role of Akt in VEGF induction of PlGF release in HUVEC.

Previously, it was shown that VEGF activates Akt (Gerber et al., 1998b), and that activation of Akt was found to suppress PlGF expression (section 3.2.8, 9 and 10). HUVEC stimulation with VEGF induced PlGF secretion from endothelial cells (Section 5.2.9). To further investigate the potential role of Akt in VEGF-mediated regulation of PlGF release, Akt-1 was knocked down in HUVEC in the presence of VEGF (20 ng/ml), or control. As demonstrated previously (Section 3.2.10), the loss of Akt-1 activity in the absence of VEGF in HUVEC significantly induced PlGF release compared with control ($p < 0.05$, $n = 3$). In the presence of VEGF, there was further ~ 2-fold increase, suggesting that VEGF regulation of PlGF expression is independent of Akt activity (Figure 5.2.10).

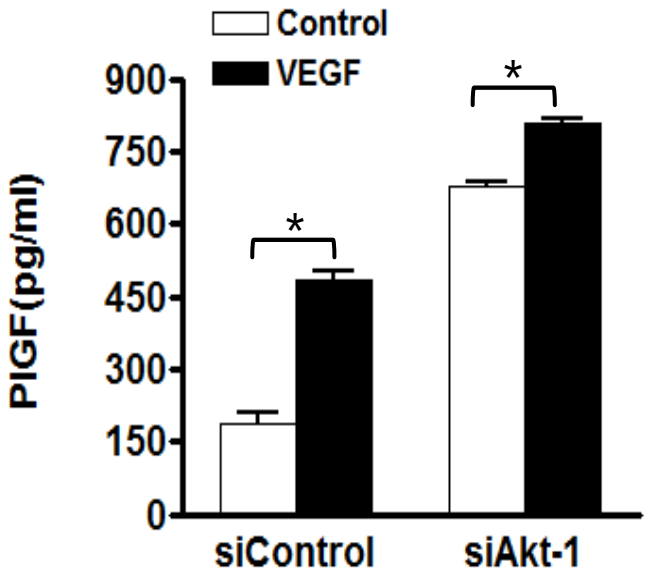


Figure 5.2.10. ***The role of Akt in VEGF-mediated PlGF release in HUVEC.*** *HUVEC were electroporated with siRNA targeted to Akt-1 or control siRNA using an Amaxa nucleofector and incubated overnight. After 24 hour incubation in growth factor-free MCDB 131 medium containing 10% FBS in the presence of VEGF (20ng/ml) or control, and PlGF was measured by ELISA in cell-conditioned medium. (*P<0.05, n=3).*

5.2.11. The role of FOXO1 in VEGF induction of PlGF release in HUVEC.

In Chapter 4, it was demonstrated that PlGF expression is modulated by FOXO1; however, FOXO1 activity is negatively regulated by VEGF (Potente et al., 2005). To examine the potential involvement of FOXO1 in VEGF-mediated regulation of PlGF expression, FOXO1 was silenced by siRNA in HUVEC overnight, followed by 24 hour incubation in the presence of VEGF (20 ng/ml) and PlGF was quantified by ELISA. As shown in Figure 5.2.11, FOXO1 knockdown, slightly reduced PlGF expression under basal conditions. In the presence of VEGF, FOXO1 knockdown had no significant effect on the stimulatory effect of VEGF on PlGF release ($p > 0.05$, $n = 3$). This suggests that VEGF-mediated PlGF release is largely independent of FOXO1 activity.

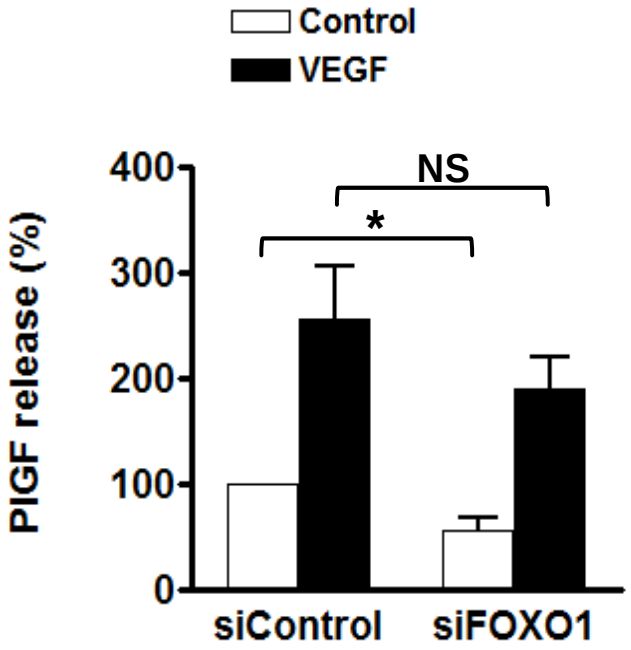


Figure 5.2.11. ***The role of the FOXO1 in VEGF-mediated PlGF release in HUVEC.*** *HUVEC were electroporated with siRNA targeted to FOXO1 or control siRNA using an Amaxa nucleofector and incubated overnight. After 24 hour incubation in growth factor-free MCDB 131 medium containing 10% FBS in the presence of VEGF (20ng/ml) or control, and PlGF was measured by ELISA in cell-conditioned medium. (*P<0.05, n=3). NS = not significant.*

5.2.12. The role of PKC in VEGF induction of PlGF release in HUVEC.

To examine the role of PKC pathway, which is also activated by VEGF (Takahashi et al., 1999) and had been implicated in VEGF-mediated regulation of PlGF expression in endothelial cells (Yao et al., 2005; Zhao et al., 2004), HUVEC were treated with GF109203X, a potent pan-PKC pharmacological inhibitor, in the presence of VEGF (20 ng/ml), or control for 24 hours and PlGF was quantified by ELISA in cell supernatants. The inhibition of PKC had no effect on PlGF release in the absence of VEGF, but VEGF-mediated up-regulation of PlGF release was significantly suppressed ($p<0.05$, $n = 3$), suggesting that VEGF regulation of PlGF expression is PKC-dependent (Figure 5.2.12). This is in line with the findings of Zao et al., (2004) who stimulated bovine microvascular endothelial cells with VEGF, in the presence or absence of GF109203X to reach the same conclusion.

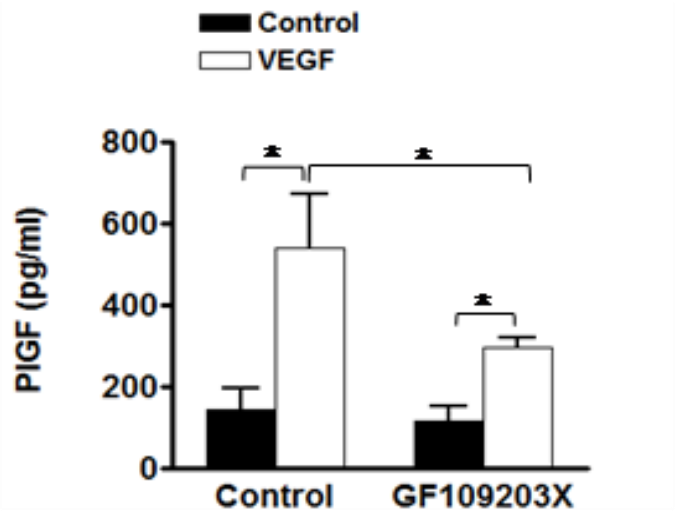


Figure 5.2.12. ***The role of the PKC signalling pathway in VEGF-mediated PlGF release in HUVEC.*** *Confluent HUVEC were treated with 5 μM of GF109309X, or control for 24 hours in MCDB 131 medium containing 10% FBS and VEGF (20 ng/ml), or control. PlGF was measured by ELISA in cell-conditioned medium. (*P<0.05, n=3).*

5.2.13. Effect of VEGFR-1 and VEGFR-2 knockdown on Akt activity in HUVEC.

In Chapter 3, the role of Akt in the regulation of PlGF expression was established. In addition, our group showed that both VEGFR-1 and VEGFR-2 mediate growth factor activation of Akt in HUVEC (Ahmad et al., 2006). This provided a rational to examine the effect of VEGFR-1 and VEGFR-2 loss on Akt activity in endothelial cells. To this end, VEGFR-1 and VEGFR-2 were knocked down by siRNA in HUVEC infected with Ad-$VEGF_{164}$, Ad-PlGF-2, or Ad-β-gal overnight. As shown by Western blotting analysis, the silencing of VEGFR-1 reduced Akt phosphorylation ($pAkt^{ser473}$) compared with VEGFR-2 knockdown, with no change in Akt expression. The reported stimulatory effect of PlGF over-expression on Akt activity (Ahmad et al., 2006; Cai et al., 2003b) was also moderately suppressed by VEGFR-1 ablation. VEGF-mediated activation of Akt, however, was not affected by the individual knockdown of either VEGFR-1 or VEGFR-2, suggesting that VEGF is capable of eliciting its biological effects via either receptor VEGFR-1 or VEGFR-2 (Figure 5.2.13). However, VEGF uses more than one receptor and potentially multiple signalling pathways to exert its biological effects. Therefore, a different approach should be sought to determine the exact role of each receptor independently in Akt activation by PlGF and VEGF.

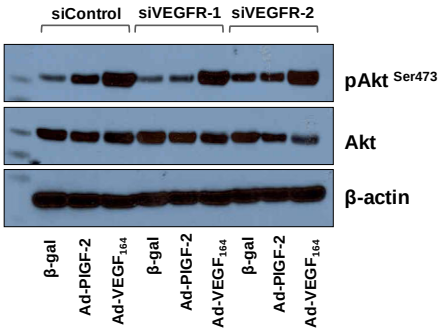


Figure 5.2.13. ***Effect of VEGFR-1 and VEGFR-2 knockdown on Akt activity in HUVEC.*** *HUVEC were electroporated with siRNA targeted to VEGFR-1, VEGFR-2, or control siRNA using an Amaxa nucleofector, and transduced with adenoviruses encoding murine PlGF-2, murine $VEGF_{164}$, or β-gal and incubated overnight. After 24 hour incubation in growth factor-free MCDB 131 medium containing 10% FBS, cell lysates were Western blotted using antibodies recognising phospho-Akt (Ser 473), Akt and β-actin. This is a representative blot of 3 independent experiments.*

5.3. Discussion

The stimulation of HUVEC with recombinant $VEGF_{165}$, or transduction with adenovirus encoding $VEGF_{164}$ significantly induced PlGF release. Data in this Chapter confirms those of other investigators (Yao et al., 2005; Zhao et al., 2004), who showed that exogenous VEGF induces PlGF production in endothelial cells. Induction of PlGF release by VEGF produced within the cell has not, however, been assessed before. VEGF clearly activates the PI3K/Akt signalling pathway, yet, promotes PlGF expression (Byzova et al., 2000; Gerber et al., 1998). Zhao et al., (2004) found that VEGF-mediated the up-regulation of PlGF in bovine microvascular endothelial cells by hyperglycaemia can be abolished using neutralising antibodies to VEGF, VEGFR-2, or pharmacological inhibitors that block the PKC signalling pathway. VEGF expression in endothelial cells promotes the phosphorylation of VEGFR-2, maintenance of endothelial cell survival and vascular homeostasis when studied in the mouse endothelial cells (Lee et al., 2007). It was therefore hypothesised that the loss of VEGF in endothelial cells might suppress PlGF expression. However, VEGF expression in endothelial cells, such as HUVEC, has been extremely difficult to detect by ELISA and qPCR. Unexpectedly, VEGF knockdown led to a significant induction of PlGF release in HUVEC. This raised further questions about the possible role of VEGF in the regulation of PlGF release in endothelial cells.

In 2006, our group demonstrated that VEGF could activate Akt via both, VEGFR-1 and VEGFR-2, through the PI3K and PKC signalling pathways (Ahmad et al., 2006). To define the mechanism of VEGF regulation of PlGF expression, the first question addressed was to delineate the separate roles of VEGFR-1 and VEGFR-2. As the data

in this Chapter show, the ablation of VEGFR-2, or inhibition of its activity using SU5416, significantly reduced VEGF-mediated PlGF release compared with control. The use of the VEGFR-2 inhibitor, SU5416, or VEGFR-2 knockdown, did not affect the level of PlGF release in the absence of exogenous VEGF, but did not inhibit recombinant $VEGF_{165}$-stimulated PlGF secretion. Whereas, the knockdown of VEGFR-1 strongly induced PlGF production in both, the presence and absence of murine PlGF-2 and $VEGF_{164}$ over-expression. These results strongly suggest that VEGFR-1 is playing a negative role in PlGF production. The observed increase in PlGF release could be explained by the displacement theory. In the presence of VEGFR-1, over-expression of murine PlGF-2 increases PlGF secretion. This might be due to the displacement of the human PlGF from endogenous VEGFR-1/s-VEGFR-1 which is then detected by human PlGF ELISA. Alternatively, there may be some cross-reactivity of murine PlGF-2 in the human PlGF ELISA leading to this result. This needs to be explored further by qPCR which would indicate whether over-expression of murine PlGF-2 leads to increased PlGF gene expression and examining levels of murine PlGF-2 production produced in transduced HUVEC and any cross-reactivity with the human ELISA. To conclude, VEGFR-1 and VEGFR-2 have different, but possibly complementary roles and there may be co-operation between the receptors in the regulation of PlGF expression in endothelial cells.

One possibility from the results obtained in Figure 5.2.6 is that VEGF may be present in endothelial cells as PlGF:VEGF heterodimer. Cao et al., (1996) have demonstrated that VEGF and PlGF preferentially form heterodimers when co-expressed within a cell. If there were a relatively small amount of VEGF present in endothelial cells compared to PlGF we predict that any VEGF would be present mostly as PlGF:VEGF heterodimers,

rather than VEGF:VEGF homodimers. To address this question, a PlGF:VEGF heterodimer sandwich ELISA was devised comprising monoclonal anti-PlGF:VEGF heterodimer, as a capture antibody and polyclonal anti-VEGF, as a detection antibody. It was postulated that the ablation of either PlGF or VEGF would lead to the formation of more PlGF:PlGF or VEGF:VEGF homodimers and cause a subsequent reduction reduction in PlGF:VEGF heterodimer levels released by endothelial cells, which we were able to detect in HUVEC supernatants at approximately 50 pg/ml using the ELISA. However, PlGF:VEGF heterodimer levels were unaltered by either PlGF or VEGF knockdown, suggesting that the preferential formation of PlGF:VEGF heterodimers is not associated with either with VEGF-mediated regulation of PlGF release, or the inability to detect VEGF secretion in HUVEC. This requires further investigation in HUVEC.

To exclude the involvement of the PI3K/Akt/FOXO1 axis in VEGF-mediated PlGF release, signalling through this pathway was manipulated in the absence or presence of VEGF, and PlGF secretion quantified by ELISA. The blockade of PI3K in HUVEC using LY294002 significantly induced PlGF in the absence of VEGF, and there was a further increase upon stimulation with VEGF, indicating that VEGF acts independently of PI3K to promote PlGF production. The same result was achieved when Akt-1 or FOXO1 were silenced in HUVEC, suggesting that both Akt-1 and FOXO1 are also not involved in VEGF-mediated increase in PlGF secretion. The inhibition of the PKC pathway, which is a target of VEGF signalling downstream of VEGFR-2 in endothelial cells, significantly suppressed PlGF induction by VEGF on the PlGF, lending further support to the role of VEGFR-2 in the mediation of VEGF regulation of PlGF. The identification of transcription factors that mediate the effects of VEGF on the PlGF

gene promoter remains a complex challenge, since the co-operation of more than one transcription factor may be involved.

The increase of PlGF release following VEGFR-1 knockdown suggested the possible presence of an autocrine loop regulating PlGF and VEGFR-1 expression, with Akt potentially acting as both a sensor and regulator in this loop. Therefore, one would speculate that, the loss of one of the ends in the loop (VEGFR-1 or PlGF) would alter Akt activity leading to an increase in the expression of the other end, thus creating a system of compensation for the loss of either, the receptor or the ligand. This may be similar to Tie2/angiopoietin system, where angiopoietin-2 (Ang-2) is induced by FOXO1 following the loss of PI3K/Akt activity (Daly et al., 2006).

Taken together, exogenous VEGF-mediated induction of PlGF release in endothelial cells is VEGFR-2 and PKC-dependent. In addition, although, it was difficult to detect endogenous VEGF in endothelial cells, the observed effects of its knockdown remain difficult to explain and further work is required to understand them.

6. Final discussion and future work.

In this thesis, the importance of the PI3K/Akt/FOXO1 signalling pathway in hyperglycaemia-mediated control of PlGF expression has been shown. A direct interaction and regulation of VEGFR-1 by FOXO1 has also been demonstrated in endothelial cells. This discovery will have major implications for pathologies characterised by endothelial dysregulation in PlGF or VEGFR-1 expression and PI3K/Akt/FOXO1 function.

VEGF regulation of PlGF release

Mechanisms of VEGF induction of PlGF release in ECs have also been investigated. Exogenous VEGF, in agreement with data previously reported, induces PlGF release in endothelial cells via the VEGFR-2/MAPK signalling axis (Zhao et al., 2004). My data showed that augmentation of PlGF expression by hyperglycaemia is independent of VEGF in EC for two reasons, 1); it was not possible to detect endogenous VEGF in EC by qPCR and ELISA. 2), if VEGF levels in HUVEC are present but undetectable, its silencing had no effect on hyperglycaemia induction of PlGF, suggesting that it is independent of VEGF. The unexpected effect of VEGF knockdown raised the possibility of a role for the naturally occurring VEGF/PlGF heterodimer, but this was also ruled out by lack of change in the levels of VEGF/PlGF heterodimer released upon the VEGF or PlGF knockdown in HUVEC. The inability to detect VEGF compounded by its ability to bind multiple receptors and activate various interlinked signalling pathways simultaneously made the definition of its exact role in the regulation PlGF release very difficult. This may be feasible by further VEGF qPCR optimisations.

The PI3K/Akt pathway and PlGF expression

Metabolic and cardiovascular disorders are characterised by endothelial dysfunction, which is characterised by a loss of PI3K/Akt signalling. PlGF expression is also up-regulated in these disorders and we carried out investigations to determine whether the two are interlinked. In these *in vitro* studies the effect of hyperglycaemia on PlGF expression in EC was examined and complementary systems to modulate the PI3K/Akt pathway activity were used. Inhibition of the PI3K/Akt pathway significantly induced PlGF expression at an mRNA and protein level, and its sustained activation had the converse effect. PlGF expression was found to be significantly higher at an mRNA and protein level in the liver and plasma, respectively, in the severely hyperglycaemic and obese ob/ob mice, compared with their lean controls. This is consistent with the finding of another study showing augmented PlGF mRNA levels in the adipose tissue of obese ob/ob compared with lean counterparts (Voros et al., 2005). PlGF mRNA in diabetic patients was also reported to be higher compared with healthy controls (Siervo et al., 2010), suggesting that increased glucose levels are the underlying cause of PlGF augmentation in these subjects. The discovery that PlGF expression is induced at an mRNA and protein level upon inhibition of PI3K and Akt may be important in understanding the pathologies associated not only with insulin resistance, but also in other diseases. PTEN mutation, resulting in a constitutively-active Akt is a frequent occurrence in cancer (Li et al., 1997), PTEN phosphorylation, promoting its antagonistic activity of PI3K was induced by hyperglycaemia in HUVEC through the tumour suppressor transcription factor, LKB1 *in vitro* (Song et al., 2007). *In vivo*, specific inhibition of PTEN expression in mice reversed the effects of hyperglycaemia (Butler et al., 2002). The benefits of PTEN activation as a strategy to inhibit PlGF–mediated inflammation in the clinic would be outweighed by the serious adverse effect

similar to that of the mutated PTEN, leading to the promotion of sustained Akt activation and cell survival, and thus cancer.

Altered Akt activity is a major feature of diverse cellular processes and diseases. Phosphorylated Akt can directly regulate the activity of its target genes by translocation to the cell nucleus or indirectly by interaction with transcription factors. In human fat and muscle cells, insulin activation of Akt mediates the promotion of glucose up-take through the translocation of GLUT4 from intracellular storage sites to cell membrane (James et al., 1988; James et al., 1989). The blockade of GLUT4 translocation by inhibition of Akt phosphorylation is considered the main cause of insulin resistance in metabolic abnormalities of type 2 diabetes. In endothelial cells, the suppression of Akt activity induces apoptosis, inflammation and abrogates NO production, key trigger events in the development of atherosclerosis. The loss of Akt-1 in atherosclerosis susceptible $ApoE^{-/-}$ deficient mice fed a Western diet, induced PlGF expression 3-fold compared with wild-type controls. These mice exhibited exacerbated atherosclerosis and coronary artery disease (Fernandez-Hernando et al., 2007). In another study, constitutively-active Akt, targeted to the endothelium in transgenic mice, led to reduced vascular lesion formation compared with control (Mukai et al., 2006). What is more interesting about this study is the fact that this effect was only partially attributed to NO loss, but predominantly to increased endothelial survival and decreased inflammatory changes. This is consistent with the role of PlGF as a promoter of inflammation in atherosclerosis (Luttun et al., 2002; Pilarczyk et al., 2008). It would be interesting to quantify the levels of PlGF in these mice to determine whether PlGF expression is correlated with the increased inflammation.

Despite the importance of this discovery, the manipulation of Akt for therapeutic purposes remains a very challenging task. Akt is localised at the centre of multiple signalling pathways and acts as a focal point at which signals converge or diverge to bring about a very diverse range of signals. How Akt selectively inhibits, or activates one single effector among hundreds is a very complex process, as each of these processes depends on the type of the stimulant, the strength and the time length of the signal. Furthermore, any Akt-activating medicinal molecule will almost ultimately favour the induction of tumour formation and growth. This difficulty warranted the identification of potential transcription factor that might directly interact and regulate PlGF expression.

FOXO1 and the regulation of PlGF expression

The discovery that PlGF release in endothelial cells is transcriptionally regulated prompted us to identify potential transcription factors downstream of Akt. The NF-κB transcription factor was a good candidate as its role in inflammatory signalling is well established and NF-κB binding sites have been reported to be in the PlGF promoter (Cramer et al., 2005). The manipulation of the activity of this transcription factor using isohelenin and a dominant-negative IKK had no effect on PlGF expression. FOXO1 is another direct downstream target of Akt and widely implicated in the regulation of metabolism, EC survival, detoxification of oxidative stress and cell cycle arrest in the endothelium (Accili and Arden, 2004; Brunet et al., 2004). Insulin/IGF–I activated Akt inactivates FOXO1 by phosphorylation and nuclear exclusion (Brunet et al., 1999). In insulin–responsive tissues, one of the major effects of hyperglycaemia-induced persistent FOXO1 nuclear localisation and blunting of insulin intracellular signalling is

the inhibition of glucose up-take, triglyceride clearance and induced glucose production (Accili and Arden, 2004). The elevation of PlGF in obese ob/ob mice associated with the suppression of the PI3K/Akt and induced phosphorylation of FoxO1 reported by other groups (Behl et al., 2009) provoked the examination of the potential role for FOXO1 in the regulation of PlGF expression in endothelial cells. In this thesis I showed not only that the silencing of FOXO1 suppressed PlGF expression, but also abrogated hyperglycaemia-induced PlGF expression *in vitro*. In addition, the demonstration that constitutively-active FOXO1 over-expression promoted PlGF expression, and this effect was not reversed by IGF-I stimulation, clearly demonstrated that the effect of hyperglycaemia is primarily attributed to persistent FOXO1 nuclear localisation. These data were corroborated by experiments performed on FVB/N mice where the transduction of adenovirus encoding constitutively-active FOXO1, significantly augmented hepatic PlGF mRNA expression and plasma protein levels. It would therefore be interesting to quantify PlGF in endothelium-specific heterozygous-FoxO1-deficient mice, a model that is already established. The performance of ChIP on HUVEC transduced with FoxO1-ADA demonstrated that FOXO1 binds to the PlGF promoter. This is in line with findings from another study showing that PlGF expression is up-regulated by FoxD1/BF-2, which is exclusively expressed by stromal cells in the embryonic renal cortex. FoxD1/BF-2 binds PlGF promoter at a HNF-3β site (Zhang et al., 2003). Similarly, the forkhead-related factor glial cell missing-1(GCM-1) was reported to regulate PlGF transcription in placental trophoblasts. The over-expression of GCM-1 prevented the inhibitory effects of hypoxia on PlGF expression (Chang et al., 2008). These findings do not, however, rule out possible indirect activation of the PlGF promoter through interaction of FOXO1 with other transcription factors. Data from this study showed that the ablation of Ets-2, that can act as a

transcriptional repressor, increased PlGF expression and the interaction between FOXO factors and members of the ETS family in the maintenance of endothelial homeostasis has been reported (De Val et al., 2008). Another member of ETS family of transcription factors is Ets-related gene (Erg), was also shown to potentially modulate PlGF expression at an mRNA and protein level (PWH unpublished data). The ideal approach to address the potential direct and in-direct interaction of FOXO1 with the PlGF promoter would be to construct an adenovirus encoding a constitutively-active FOXO1, but with a mutated DNA binding domain. The alteration of PlGF expression in HUVEC transduced with this adenovirus (or lack of it) would clarify whether FOXO1 interacts indirectly with the PlGF promoter.

FOXO1 activity and implications for obesity

FOXO1 is highly expressed in both white and brown adipose tissue. It is constitutively active and it is a major target of insulin regulation in this tissue (Nakae et al., 2003). FOXO1 is known to play an essential role in pre-adipocyte differentiation into mature adipocytes. Pre-adipocytes express very low levels of FoxO1, but as they differentiate into mature adipocytes, FoxO1 expression increases significantly. Pre-adipocytes over-expressing constitutively-active FoxO1 do not differentiate (Nakae et al., 2003). Consistent with these findings, dominant-negative FoxO1 restored pre-adipocyte differentiation in insulin receptor knockout mice (Tseng et al., 2004). Furthermore, the knockout of any components of the insulin signalling pathway impaired FoxO1-mediated pre-adipocyte differentiation, and blocking of FoxO1 expulsion from the nucleus by Akt-1 knockdown in pre-adipocytes inhibited their differentiation *in vitro* (Nakae et al., 2003). The combined weight of subcutaneous and gonadal adipose tissue

in PlGF-deficient mice on standard diet for 15 weeks was significantly lower compared with wild-type control mice, with comparable total body weight in both phenotypes (Lijnen et al., 2006). In contrast, PlGF knockout mice on high fat diet had both lower total body weight, and less fat tissue compared with wild-type controls. The authors attributed the effects seen in PlGF knockout mice to reduced angiogenesis. The finding that PlGF is increased in obese ob/ob mice which are characterised by increased levels of constitutively-active FoxO1 hints at a potential role for PlGF in pre-adipocyte differentiation and adipose tissue development. This hypothesis could be confirmed or refuted by quantification of PlGF released during the differentiation of pre-adipocytes *in vitro*. This would confirm PlGF expression changes with adipocyte differentiation, but would not directly imply a role for PlGF in this process. Modulation of FOXO1 activity in pre-adipocytes in the presence and absence of PlGF would verify whether the loss of PlGF had an effect on differentiated adipocyte number or not. However, to define the role of PlGF in disorders associated with metabolic syndrome and endothelial dysfunction, ob/ob mice could be crossed with PlGF-deficient mice. The obese and PlGF-deficient mice would help to address the question of the PlGF role in pre-adipocyte differentiation and adipose tissue development. Mechanistically, PlGF acts as a chemoattractant of pro-inflammatory VEGFR-1-expressing cells, i.e. macrophages, to sites of inflammation. In humans and rodents macrophages comprise over 40 % of total adipose tissue compared with 10% in lean controls (Weisberg et al., 2006). Hence, the provocation of the obese but PlGF deficient phenotype with a high fat diet to induce atherosclerosis could be used to determine the role of PlGF in the formation of atherosclerotic plaques and their inflammatory cell content.

PlGF and oxidative stress

Hyperglycaemia-induced oxidative stress has also been shown to contribute to endothelial dysfunction and induction of FoxO1 activity (Tanaka et al., 2009). This provided an excellent rationale to further delineate the role of FOXO1 in PlGF regulation. The marked augmentation of PlGF expression upon treatment of HUVEC with H_2O_2 and the significant inhibition of this stimulatory effect by IGF-I, suggested a role for FOXO1 in the induction of PlGF expression by oxidative stress. It is, however, worth noting that only 50% inhibition by IGF-1 points to a possible role for other FOXO members expressed by endothelial cells i.e. FOXO3a, which acts as downstream target of JNK activated by oxidative stress (Brunet et al., 2004). This could be confirmed by the treatment of HUVEC with H_2O_2 in the presence or absence of each of FOXO members separately or combined followed by PlGF quantification in cell supernatants by ELISA.

FOXO1 and the regulation of VEGFR-1 expression

The regulation of VEGFR-1 by over-expression of constitutively-active FoxO1, or siFOXO1 was reported in two unconfirmed gene array studies in endothelial cells (Daly et al., 2004; Potente et al., 2005). Using gain- and loss-of-function approaches, I demonstrated that the silencing FOXO1 in cultured HUVEC significantly modulated the expression of both membrane-bound and soluble forms of VEGFR-1. I also found that FOXO1 regulates VEGFR-1 by directly binding its promoter. It is essential to determine under what circumstances VEGFR-1 is regulated by FOXO1 and whether its expression changes in ob/ob mice. These data tie in very well with the discovery that the knockdown of PlGF or VEGFR-1 in endothelial cells suppresses Akt activity, and

that loss of PlGF induces the expression of its receptor, VEGFR-1, and vice versa, indicating the existence of an autocrine loop, at the centre of which Akt acts as sensor, thus, regulating the expression of both, the receptor and the ligand. This is an important finding given the essential role of Akt in the regulation of endothelial cell biology in angiogenesis and homeostasis. Evaluation of Akt activity in PlGF knockout mice, would be very interesting since the induction of angiogenesis in these mice was compromised compared to wild-type animals (Lijnen et al., 2006). The functional significance of this loop requires further investigation *in vivo*.

Final conclusion

The discovery that in endothelial dysfunction, a phenomenon that precedes multiple metabolic and cardiovascular disorders, the blunting of PI3K/Akt/FOXO1 signalling pathways in EC induces the expression of the pro-inflammatory growth factor PlGF, could provide a target for therapies aimed at suppression of inflammation in these disorders. In addition, the finding that FOXO1 induces VEGFR-1 expression promotes FOXO1 as an ideal target for manipulation in pathologies characterised by up-regulation of VEGFR-1 and PlGF.

.

7. REFERENCES

ABID, M. R., GUO, S., MINAMI, T., SPOKES, K. C., UEKI, K., SKURK, C., WALSH, K. & AIRD, W. C. 2004. Vascular endothelial growth factor activates PI3K/Akt/forkhead signaling in endothelial cells. *Arterioscler Thromb Vasc Biol,* 24**,** 294-300.

ABID, M. R., SHIH, S. C., OTU, H. H., SPOKES, K. C., OKADA, Y., CURIEL, D. T., MINAMI, T. & AIRD, W. C. 2006. A novel class of vascular endothelial growth factor-responsive genes that require forkhead activity for expression. *J Biol Chem,* 281**,** 35544-53.

ABID, M. R., YANO, K., GUO, S., PATEL, V. I., SHRIKHANDE, G., SPOKES, K. C., FERRAN, C. & AIRD, W. C. 2005. Forkhead transcription factors inhibit vascular smooth muscle cell proliferation and neointimal hyperplasia. *J Biol Chem,* 280**,** 29864-73.

ACCILI, D. & ARDEN, K. C. 2004. FoxOs at the crossroads of cellular metabolism, differentiation, and transformation. *Cell,* 117**,** 421-6.

ACHEN, M. G., JELTSCH, M., KUKK, E., MAKINEN, T., VITALI, A., WILKS, A. F., ALITALO, K. & STACKER, S. A. 1998. Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). *Proc Natl Acad Sci U S A,* 95**,** 548-53.

ADAMIS, A. P., MILLER, J. W., BERNAL, M. T., D'AMICO, D. J., FOLKMAN, J., YEO, T. K. & YEO, K. T. 1994. Increased vascular endothelial growth factor levels in the vitreous of eyes with proliferative diabetic retinopathy. *Am J Ophthalmol,* 118**,** 445-50.

ADINI, A., KORNAGA, T., FIROOZBAKHT, F. & BENJAMIN, L. E. 2002. Placental growth factor is a survival factor for tumor endothelial cells and macrophages. *Cancer Res,* 62**,** 2749-52.

AHMAD, S. & AHMED, A. 2004. Elevated placental soluble vascular endothelial growth factor receptor-1 inhibits angiogenesis in preeclampsia. *Circ Res,* 95**,** 884-91.

AHMAD, S., HEWETT, P. W., WANG, P., AL-ANI, B., CUDMORE, M., FUJISAWA, T., HAIGH, J. J., LE NOBLE, F., WANG, L., MUKHOPADHYAY, D. & AHMED, A. 2006. Direct evidence for endothelial vascular endothelial growth factor receptor-1 function in nitric oxide-mediated angiogenesis. *Circ Res,* 99**,** 715-22.

AHMED, A., LI, X. F., DUNK, C., WHITTLE, M. J., RUSHTON, D. I. & ROLLASON, T. 1995. Colocalisation of vascular endothelial growth factor and its Flt-1 receptor in human placenta. *Growth Factors,* 12**,** 235-43.

AIELLO, L. P., AVERY, R. L., ARRIGG, P. G., KEYT, B. A., JAMPEL, H. D., SHAH, S. T., PASQUALE, L. R., THIEME, H., IWAMOTO, M. A., PARK, J. E. & ET AL. 1994. Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *N Engl J Med,* 331**,** 1480-7.

AL-ANI, B., HEWETT, P. W., CUDMORE, M. J., FUJISAWA, T., SAIFEDDINE, M., WILLIAMS, H., RAMMA, W., SISSAOUI, S., JAYARAMAN, P. S., OHBA, M., AHMAD, S., HOLLENBERG, M. D. & AHMED, A. 2010. Activation of proteinase-activated receptor 2 stimulates soluble vascular endothelial growth factor receptor 1 release via epidermal growth factor receptor transactivation in endothelial cells. *Hypertension,* 55**,** 689-97.

ALESSI, D. R., JAMES, S. R., DOWNES, C. P., HOLMES, A. B., GAFFNEY, P. R., REESE, C. B. & COHEN, P. 1997. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Curr Biol,* 7**,** 261-9.

ALTOMARE, D. A., LYONS, G. E., MITSUUCHI, Y., CHENG, J. Q. & TESTA, J. R. 1998. Akt2 mRNA is highly expressed in embryonic brown fat and the AKT2 kinase is activated by insulin. *Oncogene,* 16**,** 2407-11.

ALTOMONTE, J., CONG, L., HARBARAN, S., RICHTER, A., XU, J., MESECK, M. & DONG, H. H. 2004. Foxo1 mediates insulin action on apoC-III and triglyceride metabolism. *J Clin Invest,* 114**,** 1493-503.

ALVAREZ, B., MARTINEZ, A. C., BURGERING, B. M. & CARRERA, A. C. 2001. Forkhead transcription factors contribute to execution of the mitotic programme in mammals. *Nature,* 413**,** 744-7.

ANDERSON, M. J., VIARS, C. S., CZEKAY, S., CAVENEE, W. K. & ARDEN, K. C. 1998. Cloning and characterization of three human forkhead genes that comprise an FKHR-like gene subfamily. *Genomics,* 47**,** 187-99.

AOKI, M., JIANG, H. & VOGT, P. K. 2004. Proteasomal degradation of the FoxO1 transcriptional regulator in cells transformed by the P3k and Akt oncoproteins. *Proc Natl Acad Sci U S A,* 101**,** 13613-7.

ARAKI, E., LIPES, M. A., PATTI, M. E., BRUNING, J. C., HAAG, B., 3RD, JOHNSON, R. S. & KAHN, C. R. 1994. Alternative pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene. *Nature,* 372**,** 186-90.

ARFKEN, C. L., RENO, P. L., SANTIAGO, J. V. & KLEIN, R. 1998. Development of proliferative diabetic retinopathy in African-Americans and whites with type 1 diabetes. *Diabetes Care,* 21**,** 792-5.

ARMONI, M., HAREL, C., KARNI, S., CHEN, H., BAR-YOSEPH, F., VER, M. R., QUON, M. J. & KARNIELI, E. 2006. FOXO1 represses peroxisome proliferator-activated receptor-gamma1 and -gamma2 gene promoters in primary adipocytes. A novel paradigm to increase insulin sensitivity. *J Biol Chem,* 281**,** 19881-91.

ARMONI, M., KRITZ, N., HAREL, C., BAR-YOSEPH, F., CHEN, H., QUON, M. J. & KARNIELI, E. 2003. Peroxisome proliferator-activated receptor-gamma represses GLUT4 promoter activity in primary adipocytes, and rosiglitazone alleviates this effect. *J Biol Chem,* 278**,** 30614-23.

AUTIERO, M., WALTENBERGER, J., COMMUNI, D., KRANZ, A., MOONS, L., LAMBRECHTS, D., KROLL, J., PLAISANCE, S., DE MOL, M., BONO, F., KLICHE, S., FELLBRICH, G., BALLMER-HOFER, K., MAGLIONE, D., MAYR-BEYRLE, U., DEWERCHIN, M., DOMBROWSKI, S., STANIMIROVIC, D., VAN HUMMELEN, P., DEHIO, C., HICKLIN, D. J., PERSICO, G., HERBERT, J. M., COMMUNI, D., SHIBUYA, M., COLLEN, D., CONWAY, E. M. & CARMELIET, P. 2003. Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. *Nat Med,* 9**,** 936-43.

BAILEY, C. J., FLATT, P. R. & ATKINS, T. W. 1982. Influence of genetic background and age on the expression of the obese hyperglycaemic syndrome in Aston ob/ob mice. *Int J Obes,* 6**,** 11-21.

BAIS, C., WU, X., YAO, J., YANG, S., CRAWFORD, Y., MCCUTCHEON, K., TAN, C., KOLUMAM, G., VERNES, J. M., EASTHAM-ANDERSON, J., HAUGHNEY, P., KOWANETZ, M., HAGENBEEK, T., KASMAN, I., RESLAN, H. B., ROSS, J., VAN BRUGGEN, N., CARANO, R. A., MENG, Y. J., HONGO, J. A., STEPHAN, J. P., SHIBUYA, M. & FERRARA, N. 2010. PlGF blockade does not inhibit angiogenesis during primary tumor growth. *Cell,* 141**,** 166-77.

BAKER, R. G., HAYDEN, M. S. & GHOSH, S. 2011. NF-kappaB, inflammation, and metabolic disease. *Cell Metab,* 13**,** 11-22.

BALLETSHOFER, B. M., RITTIG, K., ENDERLE, M. D., VOLK, A., MAERKER, E., JACOB, S., MATTHAEI, S., RETT, K. & HARING, H. U. 2000. Endothelial dysfunction is detectable in young normotensive first-degree relatives of subjects with type 2 diabetes in association with insulin resistance. *Circulation,* 101**,** 1780-4.

BARLEON, B., SOZZANI, S., ZHOU, D., WEICH, H. A., MANTOVANI, A. & MARME, D. 1996. Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. *Blood,* 87**,** 3336-43.

BARON, A. D. & CLARK, M. G. 1997. Role of blood flow in the regulation of muscle glucose uptake. *Annu Rev Nutr,* 17**,** 487-99.

BARTHEL, A., SCHMOLL, D. & UNTERMAN, T. G. 2005. FoxO proteins in insulin action and metabolism. *Trends Endocrinol Metab,* 16**,** 183-9.

BATES, D. O., CUI, T. G., DOUGHTY, J. M., WINKLER, M., SUGIONO, M., SHIELDS, J. D., PEAT, D., GILLATT, D. & HARPER, S. J. 2002. VEGF165b, an inhibitory splice variant of vascular endothelial growth factor, is down-regulated in renal cell carcinoma. *Cancer Res,* 62**,** 4123-31.

BAUMGARTNER-PARZER, S. M., WAGNER, L., PETTERMANN, M., GRILLARI, J., GESSL, A. & WALDHAUSL, W. 1995. High-glucose--triggered apoptosis in cultured endothelial cells. *Diabetes,* 44**,** 1323-7.

BAXTER, R. C. & TWIGG, S. M. 2009. Actions of IGF binding proteins and related proteins in adipose tissue. *Trends Endocrinol Metab,* 20**,** 499-505.

BECK, H., ACKER, T., PUSCHEL, A. W., FUJISAWA, H., CARMELIET, P. & PLATE, K. H. 2002. Cell type-specific expression of neuropilins in an MCA-occlusion model in mice suggests a potential role in post-ischemic brain remodeling. *J Neuropathol Exp Neurol,* 61**,** 339-50.

BEHL, Y., KROTHAPALLI, P., DESTA, T., ROY, S. & GRAVES, D. T. 2009. FOXO1 plays an important role in enhanced microvascular cell apoptosis and microvascular cell loss in type 1 and type 2 diabetic rats. *Diabetes,* 58**,** 917-25.

BELLACOSA, A., FRANKE, T. F., GONZALEZ-PORTAL, M. E., DATTA, K., TAGUCHI, T., GARDNER, J., CHENG, J. Q., TESTA, J. R. & TSICHLIS, P. N. 1993. Structure, expression and chromosomal mapping of c-akt: relationship to v-akt and its implications. *Oncogene,* 8**,** 745-54.

BELLACOSA, A., TESTA, J. R., STAAL, S. P. & TSICHLIS, P. N. 1991. A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. *Science,* 254**,** 274-7.

BELLIK, L., VINCI, M. C., FILIPPI, S., LEDDA, F. & PARENTI, A. 2005. Intracellular pathways triggered by the selective FLT-1-agonist placental growth factor in vascular smooth muscle cells exposed to hypoxia. *Br J Pharmacol,* 146**,** 568-75.

BEN-AV, P., CROFFORD, L. J., WILDER, R. L. & HLA, T. 1995. Induction of vascular endothelial growth factor expression in synovial fibroblasts by prostaglandin E and interleukin-1: a potential mechanism for inflammatory angiogenesis. *FEBS Lett,* 372**,** 83-7.

BERGMANN, A., AHMAD, S., CUDMORE, M., GRUBER, A. D., WITTSCHEN, P., LINDENMAIER, W., CHRISTOFORI, G., GROSS, V., GONZALVES, A., GRONE, H. J., AHMED, A. & WEICH, H. A. 2010. Reduction of circulating soluble Flt-1 alleviates preeclampsia-like symptoms in a mouse model. *J Cell Mol Med,* 14**,** 1857-67.

BERRA, E., GINOUVES, A. & POUYSSEGUR, J. 2006. The hypoxia-inducible-factor hydroxylases bring fresh air into hypoxia signalling. *EMBO Rep,* 7**,** 41-5.

BHARDWAJ, S., ROY, H., HEIKURA, T. & YLA-HERTTUALA, S. 2005. VEGF-A, VEGF-D and VEGF-D(DeltaNDeltaC) induced intimal hyperplasia in carotid arteries. *Eur J Clin Invest,* 35**,** 669-76.

BIGGS, W. H., 3RD, MEISENHELDER, J., HUNTER, T., CAVENEE, W. K. & ARDEN, K. C. 1999. Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1. *Proc Natl Acad Sci U S A,* 96**,** 7421-6.

BIRNBAUM, M. J. 1989. Identification of a novel gene encoding an insulin-responsive glucose transporter protein. *Cell,* 57**,** 305-15.

BJORNHOLM, M., KAWANO, Y., LEHTIHET, M. & ZIERATH, J. R. 1997. Insulin receptor substrate-1 phosphorylation and phosphatidylinositol 3-kinase activity in skeletal muscle from NIDDM subjects after in vivo insulin stimulation. *Diabetes,* 46**,** 524-7.

BLOOM, S. 2005. Belgian scientists awarded top honors. *J Clin Invest,* 115**,** 1106-7.

BODINE, S. C., STITT, T. N., GONZALEZ, M., KLINE, W. O., STOVER, G. L., BAUERLEIN, R., ZLOTCHENKO, E., SCRIMGEOUR, A., LAWRENCE, J. C., GLASS, D. J. & YANCOPOULOS, G. D. 2001. Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. *Nat Cell Biol,* 3**,** 1014-9.

BODOLAY, E., KOCH, A. E., KIM, J., SZEGEDI, G. & SZEKANECZ, Z. 2002. Angiogenesis and chemokines in rheumatoid arthritis and other systemic inflammatory rheumatic diseases. *J Cell Mol Med,* 6**,** 357-76.

BOIS, P. R. & GROSVELD, G. C. 2003. FKHR (FOXO1a) is required for myotube fusion of primary mouse myoblasts. *Embo J,* 22**,** 1147-57.

BOTTOMLEY, M. J., WEBB, N. J., WATSON, C. J., HOLT, L., BUKHARI, M., DENTON, J., FREEMONT, A. J. & BRENCHLEY, P. E. 2000. Placenta growth factor (PlGF) induces vascular endothelial growth factor (VEGF) secretion from mononuclear cells and is co-expressed with VEGF in synovial fluid. *Clin Exp Immunol,* 119**,** 182-8.

BOURA, E., SILHAN, J., HERMAN, P., VECER, J., SULC, M., TEISINGER, J., OBSILOVA, V. & OBSIL, T. 2007. Both the N-terminal loop and wing W2 of the forkhead domain of transcription factor Foxo4 are important for DNA binding. *J Biol Chem,* 282**,** 8265-75.

BRENKMAN, A. B., DE KEIZER, P. L., VAN DEN BROEK, N. J., JOCHEMSEN, A. G. & BURGERING, B. M. 2008. Mdm2 induces mono-ubiquitination of FOXO4. *PLoS One,* 3**,** e2819.

BRUNET, A., BONNI, A., ZIGMOND, M. J., LIN, M. Z., JUO, P., HU, L. S., ANDERSON, M. J., ARDEN, K. C., BLENIS, J. & GREENBERG, M. E. 1999. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell,* 96**,** 857-68.

BRUNET, A., SWEENEY, L. B., STURGILL, J. F., CHUA, K. F., GREER, P. L., LIN, Y., TRAN, H., ROSS, S. E., MOSTOSLAVSKY, R., COHEN, H. Y., HU, L. S., CHENG, H. L., JEDRYCHOWSKI, M. P., GYGI, S. P., SINCLAIR, D. A., ALT, F. W. & GREENBERG, M. E. 2004. Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. *Science,* 303**,** 2011-5.

BURGERING, B. M. 2008. A brief introduction to FOXOlogy. *Oncogene,* 27**,** 2258-62.

BURGERING, B. M. & MEDEMA, R. H. 2003. Decisions on life and death: FOXO Forkhead transcription factors are in command when PKB/Akt is off duty. *J Leukoc Biol,* 73**,** 689-701.

BUTLER, A. A. & LEROITH, D. 2001. Minireview: tissue-specific versus generalized gene targeting of the igf1 and igf1r genes and their roles in insulin-like growth factor physiology. *Endocrinology,* 142**,** 1685-8.

BUTLER, M., MCKAY, R. A., POPOFF, I. J., GAARDE, W. A., WITCHELL, D., MURRAY, S. F., DEAN, N. M., BHANOT, S. & MONIA, B. P. 2002. Specific inhibition of PTEN expression reverses hyperglycemia in diabetic mice. *Diabetes,* 51**,** 1028-34.

BYZOVA, T. V., GOLDMAN, C. K., PAMPORI, N., THOMAS, K. A., BETT, A., SHATTIL, S. J. & PLOW, E. F. 2000. A mechanism for modulation of cellular responses to VEGF: activation of the integrins. *Mol Cell,* 6**,** 851-60.

CABALLERO, A. E., BOUSQUET-SANTOS, K., ROBLES-OSORIO, L., MONTAGNANI, V., SOODINI, G., PORRAMATIKUL, S., HAMDY, O., NOBREGA, A. C. & HORTON, E. S. 2008. Overweight Latino children and adolescents have marked endothelial dysfunction and subclinical vascular inflammation in association with excess body fat and insulin resistance. *Diabetes Care,* 31**,** 576-82.

CAI, D., DHE-PAGANON, S., MELENDEZ, P. A., LEE, J. & SHOELSON, S. E. 2003a. Two new substrates in insulin signaling, IRS5/DOK4 and IRS6/DOK5. *J Biol Chem,* 278**,** 25323-30.

CAI, J., AHMAD, S., JIANG, W. G., HUANG, J., KONTOS, C. D., BOULTON, M. & AHMED, A. 2003b. Activation of vascular endothelial growth factor receptor-1 sustains angiogenesis and Bcl-2 expression via the phosphatidylinositol 3-kinase pathway in endothelial cells. *Diabetes,* 52**,** 2959-68.

CAO, Y., CHEN, H., ZHOU, L., CHIANG, M. K., ANAND-APTE, B., WEATHERBEE, J. A., WANG, Y., FANG, F., FLANAGAN, J. G. & TSANG, M. L. 1996. Heterodimers of placenta growth factor/vascular endothelial growth factor. Endothelial activity, tumor cell expression, and high affinity binding to Flk-1/KDR. *J Biol Chem,* 271**,** 3154-62.

CARDONE, M. H., ROY, N., STENNICKE, H. R., SALVESEN, G. S., FRANKE, T. F., STANBRIDGE, E., FRISCH, S. & REED, J. C. 1998. Regulation of cell death protease caspase-9 by phosphorylation. *Science,* 282**,** 1318-21.

CARMELIET, P. 2000. Mechanisms of angiogenesis and arteriogenesis. *Nat Med,* 6**,** 389-95.

CARMELIET, P., FERREIRA, V., BREIER, G., POLLEFEYT, S., KIECKENS, L., GERTSENSTEIN, M., FAHRIG, M., VANDENHOECK, A., HARPAL, K., EBERHARDT, C., DECLERCQ, C., PAWLING, J., MOONS, L., COLLEN, D., RISAU, W. & NAGY, A. 1996. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature,* 380**,** 435-9.

CARMELIET, P., MOONS, L., LUTTUN, A., VINCENTI, V., COMPERNOLLE, V., DE MOL, M., WU, Y., BONO, F., DEVY, L., BECK, H., SCHOLZ, D., ACKER, T., DIPALMA, T., DEWERCHIN, M., NOEL, A., STALMANS, I., BARRA, A., BLACHER, S., VANDENDRIESSCHE, T., PONTEN, A., ERIKSSON, U., PLATE, K. H., FOIDART, J. M., SCHAPER, W., CHARNOCK-JONES, D. S., HICKLIN, D. J., HERBERT, J. M., COLLEN, D. & PERSICO, M. G. 2001. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. *Nat Med,* 7**,** 575-83.

CARMELIET, P., NG, Y. S., NUYENS, D., THEILMEIER, G., BRUSSELMANS, K., CORNELISSEN, I., EHLER, E., KAKKAR, V. V., STALMANS, I., MATTOT, V., PERRIARD, J. C., DEWERCHIN, M., FLAMENG, W., NAGY, A., LUPU, F., MOONS, L., COLLEN, D., D'AMORE, P. A. & SHIMA, D. T. 1999. Impaired myocardial angiogenesis and ischemic cardiomyopathy in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. *Nat Med,* 5**,** 495-502.

CARMELIET, P. & STORKEBAUM, E. 2002. Vascular and neuronal effects of VEGF in the nervous system: implications for neurological disorders. *Semin Cell Dev Biol,* 13**,** 39-53.

CASANOVAS, O., HICKLIN, D. J., BERGERS, G. & HANAHAN, D. 2005. Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors. *Cancer Cell,* 8**,** 299-309.

CASTRILLON, D. H., MIAO, L., KOLLIPARA, R., HORNER, J. W. & DEPINHO, R. A. 2003. Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. *Science,* 301**,** 215-8.

CHANG, M., MUKHERJEA, D., GOBBLE, R. M., GROESCH, K. A., TORRY, R. J. & TORRY, D. S. 2008. Glial cell missing 1 regulates placental growth factor (PGF) gene transcription in human trophoblast. *Biol Reprod,* 78**,** 841-51.

CHEN, C. N., HSIEH, F. J., CHENG, Y. M., CHENG, W. F., SU, Y. N., CHANG, K. J. & LEE, P. H. 2004. The significance of placenta growth factor in angiogenesis and clinical outcome of human gastric cancer. *Cancer Lett,* 213**,** 73-82.

CHEN, J., SOMANATH, P. R., RAZORENOVA, O., CHEN, W. S., HAY, N., BORNSTEIN, P. & BYZOVA, T. V. 2005. Akt1 regulates pathological angiogenesis, vascular maturation and permeability in vivo. *Nat Med,* 11**,** 1188-96.

CHEN, S., APOSTOLOVA, M. D., CHERIAN, M. G. & CHAKRABARTI, S. 2000. Interaction of endothelin-1 with vasoactive factors in mediating glucose-induced increased permeability in endothelial cells. *Lab Invest,* 80**,** 1311-21.

CHLENCH, S., MECHA DISASSA, N., HOHBERG, M., HOFFMANN, C., POHLKAMP, T., BEYER, G., BONGRAZIO, M., DA SILVA-AZEVEDO, L., BAUM, O., PRIES, A. R. & ZAKRZEWICZ, A. 2007. Regulation of Foxo-1 and the angiopoietin-2/Tie2 system by shear stress. *FEBS Lett,* 581**,** 673-80.

CHO, H., MU, J., KIM, J. K., THORVALDSEN, J. L., CHU, Q., CRENSHAW, E. B., 3RD, KAESTNER, K. H., BARTOLOMEI, M. S., SHULMAN, G. I. & BIRNBAUM, M. J. 2001a. Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). *Science,* 292**,** 1728-31.

CHO, H., THORVALDSEN, J. L., CHU, Q., FENG, F. & BIRNBAUM, M. J. 2001b. Akt1/PKBalpha is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. *J Biol Chem,* 276**,** 38349-52.

CHO, N. K., KEYES, L., JOHNSON, E., HELLER, J., RYNER, L., KARIM, F. & KRASNOW, M. A. 2002. Developmental control of blood cell migration by the Drosophila VEGF pathway. *Cell,* 108**,** 865-76.

CIECHANOVER, A. 1998. The ubiquitin-proteasome pathway: on protein death and cell life. *Embo J,* 17**,** 7151-60.

CLARK, K. L., HALAY, E. D., LAI, E. & BURLEY, S. K. 1993. Co-crystal structure of the HNF-3/fork head DNA-recognition motif resembles histone H5. *Nature,* 364**,** 412-20.

CLAUSS, M., GERLACH, M., GERLACH, H., BRETT, J., WANG, F., FAMILLETTI, P. C., PAN, Y. C., OLANDER, J. V., CONNOLLY, D. T. & STERN, D. 1990. Vascular permeability factor: a tumor-derived polypeptide that induces endothelial cell and monocyte procoagulant activity, and promotes monocyte migration. *J Exp Med,* 172**,** 1535-45.

COLLAS, P. & DAHL, J. A. 2008. Chop it, ChIP it, check it: the current status of chromatin immunoprecipitation. *Front Biosci,* 13**,** 929-43.

COURTENAY, J. S., DALLMAN, M. J., DAYAN, A. D., MARTIN, A. & MOSEDALE, B. 1980. Immunisation against heterologous type II collagen induces arthritis in mice. *Nature,* 283**,** 666-8.

CRAMER, M., NAGY, I., MURPHY, B. J., GASSMANN, M., HOTTIGER, M. O., GEORGIEV, O. & SCHAFFNER, W. 2005. NF-kappaB contributes to transcription of placenta growth factor and interacts with metal responsive transcription factor-1 in hypoxic human cells. *Biol Chem,* 386**,** 865-72.

CUNNINGHAM, S. A., WAXHAM, M. N., ARRATE, P. M. & BROCK, T. A. 1995. Interaction of the Flt-1 tyrosine kinase receptor with the p85 subunit of phosphatidylinositol 3-kinase. Mapping of a novel site involved in binding. *J Biol Chem,* 270**,** 20254-7.

DAGHER, Z., PARK, Y. S., ASNAGHI, V., HOEHN, T., GERHARDINGER, C. & LORENZI, M. 2004. Studies of rat and human retinas predict a role for the polyol pathway in human diabetic retinopathy. *Diabetes,* 53**,** 2404-11.

DAITOKU, H., HATTA, M., MATSUZAKI, H., ARATANI, S., OHSHIMA, T., MIYAGISHI, M., NAKAJIMA, T. & FUKAMIZU, A. 2004. Silent information regulator 2 potentiates Foxo1-mediated transcription through its deacetylase activity. *Proc Natl Acad Sci U S A,* 101**,** 10042-7.

DAITOKU, H., YAMAGATA, K., MATSUZAKI, H., HATTA, M. & FUKAMIZU, A. 2003. Regulation of PGC-1 promoter activity by protein kinase B and the forkhead transcription factor FKHR. *Diabetes,* 52**,** 642-9.

DALY, C., PASNIKOWSKI, E., BUROVA, E., WONG, V., ALDRICH, T. H., GRIFFITHS, J., IOFFE, E., DALY, T. J., FANDL, J. P., PAPADOPOULOS, N., MCDONALD, D. M., THURSTON, G., YANCOPOULOS, G. D. & RUDGE, J. S. 2006. Angiopoietin-2 functions as an autocrine protective factor in stressed endothelial cells. *Proc Natl Acad Sci U S A,* 103**,** 15491-6.

DALY, C., WONG, V., BUROVA, E., WEI, Y., ZABSKI, S., GRIFFITHS, J., LAI, K. M., LIN, H. C., IOFFE, E., YANCOPOULOS, G. D. & RUDGE, J. S. 2004. Angiopoietin-1 modulates endothelial cell function and gene expression via the transcription factor FKHR (FOXO1). *Genes Dev,* 18**,** 1060-71.

DATTA, K., BELLACOSA, A., CHAN, T. O. & TSICHLIS, P. N. 1996. Akt is a direct target of the phosphatidylinositol 3-kinase. Activation by growth factors, v-src and v-Ha-ras, in Sf9 and mammalian cells. *J Biol Chem,* 271**,** 30835-9.

DATTA, S. R., BRUNET, A. & GREENBERG, M. E. 1999. Cellular survival: a play in three Akts. *Genes Dev,* 13**,** 2905-27.

DATTA, S. R., DUDEK, H., TAO, X., MASTERS, S., FU, H., GOTOH, Y. & GREENBERG, M. E. 1997. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell,* 91**,** 231-41.

DAVIS-SMYTH, T., PRESTA, L. G. & FERRARA, N. 1998. Mapping the charged residues in the second immunoglobulin-like domain of the vascular endothelial growth factor/placenta growth factor receptor Flt-1 required for binding and structural stability. *J Biol Chem,* 273**,** 3216-22.

DAVIS, R. J., D'CRUZ, C. M., LOVELL, M. A., BIEGEL, J. A. & BARR, F. G. 1994. Fusion of PAX7 to FKHR by the variant t(1;13)(p36;q14) translocation in alveolar rhabdomyosarcoma. *Cancer Res,* 54**,** 2869-72.

DE CATERINA, R. 2000. Endothelial dysfunctions: common denominators in vascular disease. *Curr Opin Lipidol,* 11**,** 9-23.

DE VAL, S., CHI, N. C., MEADOWS, S. M., MINOVITSKY, S., ANDERSON, J. P., HARRIS, I. S., EHLERS, M. L., AGARWAL, P., VISEL, A., XU, S. M., PENNACCHIO, L. A., DUBCHAK, I., KRIEG, P. A., STAINIER, D. Y. & BLACK, B. L. 2008. Combinatorial regulation of endothelial gene expression by ets and forkhead transcription factors. *Cell,* 135**,** 1053-64.

DE VRIES, C., ESCOBEDO, J. A., UENO, H., HOUCK, K., FERRARA, N. & WILLIAMS, L. T. 1992. The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science,* 255**,** 989-91.

DEKKER, J. M., GIRMAN, C., RHODES, T., NIJPELS, G., STEHOUWER, C. D., BOUTER, L. M. & HEINE, R. J. 2005. Metabolic syndrome and 10-year cardiovascular disease risk in the Hoorn Study. *Circulation,* 112**,** 666-73.

DEPOIX, C., TEE, M. K. & TAYLOR, R. N. 2011. Molecular regulation of human placental growth factor (PlGF) gene expression in placental villi and trophoblast cells is mediated via the protein kinase a pathway. *Reprod Sci,* 18**,** 219-28.

DIJKERS, P. F., MEDEMA, R. H., PALS, C., BANERJI, L., THOMAS, N. S., LAM, E. W., BURGERING, B. M., RAAIJMAKERS, J. A., LAMMERS, J. W., KOENDERMAN, L. & COFFER, P. J. 2000. Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of p27(KIP1). *Mol Cell Biol,* 20**,** 9138-48.

DIKOV, M. M., OHM, J. E., RAY, N., TCHEKNEVA, E. E., BURLISON, J., MOGHANAKI, D., NADAF, S. & CARBONE, D. P. 2005. Differential roles of vascular endothelial growth factor receptors 1 and 2 in dendritic cell differentiation. *J Immunol,* 174**,** 215-22.

DISALVO, J., BAYNE, M. L., CONN, G., KWOK, P. W., TRIVEDI, P. G., SODERMAN, D. D., PALISI, T. M., SULLIVAN, K. A. & THOMAS, K. A. 1995. Purification and characterization of a naturally occurring vascular endothelial growth factor.placenta growth factor heterodimer. *J Biol Chem,* 270**,** 7717-23.

DOR, Y., PORAT, R. & KESHET, E. 2001. Vascular endothelial growth factor and vascular adjustments to perturbations in oxygen homeostasis. *Am J Physiol Cell Physiol,* 280**,** C1367-74.

DOWELL, P., OTTO, T. C., ADI, S. & LANE, M. D. 2003. Convergence of peroxisome proliferator-activated receptor gamma and Foxo1 signaling pathways. *J Biol Chem,* 278**,** 45485-91.

DU, X. L., EDELSTEIN, D., ROSSETTI, L., FANTUS, I. G., GOLDBERG, H., ZIYADEH, F., WU, J. & BROWNLEE, M. 2000. 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**,** 12222-6.

DU, X. L., SUI, G. Z., STOCKKLAUSER-FARBER, K., WEISS, J., ZINK, S., SCHWIPPERT, B., WU, Q. X., TSCHOPE, D. & ROSEN, P. 1998. Introduction of apoptosis by high proinsulin and glucose in cultured human umbilical vein endothelial cells is mediated by reactive oxygen species. *Diabetologia,* 41**,** 249-56.

DUIVENVOORDEN, I., TEUSINK, B., RENSEN, P. C., ROMIJN, J. A., HAVEKES, L. M. & VOSHOL, P. J. 2005. Apolipoprotein C3 deficiency results in diet-induced obesity and aggravated insulin resistance in mice. *Diabetes,* 54**,** 664-71.

DVORAK, H. F., BROWN, L. F., DETMAR, M. & DVORAK, A. M. 1995. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am J Pathol,* 146**,** 1029-39.

ELCHEBLY, M., PAYETTE, P., MICHALISZYN, E., CROMLISH, W., COLLINS, S., LOY, A. L., NORMANDIN, D., CHENG, A., HIMMS-HAGEN, J., CHAN, C. C., RAMACHANDRAN, C., GRESSER, M. J., TREMBLAY, M. L. & KENNEDY, B. P. 1999. Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene. *Science,* 283**,** 1544-8.

ELICEIRI, B. P., PAUL, R., SCHWARTZBERG, P. L., HOOD, J. D., LENG, J. & CHERESH, D. A. 1999. Selective requirement for Src kinases during VEGF-induced angiogenesis and vascular permeability. *Mol Cell,* 4**,** 915-24.

EMANUELLI, B., PERALDI, P., FILLOUX, C., CHAVEY, C., FREIDINGER, K., HILTON, D. J., HOTAMISLIGIL, G. S. & VAN OBBERGHEN, E. 2001. SOCS-3 inhibits insulin signaling and is up-regulated in response to tumor necrosis factor-alpha in the adipose tissue of obese mice. *J Biol Chem,* 276**,** 47944-9.

ENGERMAN, R. L. & KERN, T. S. 1995. Retinopathy in animal models of diabetes. *Diabetes Metab Rev,* 11**,** 109-20.

ERIKSSON, A., CAO, R., PAWLIUK, R., BERG, S. M., TSANG, M., ZHOU, D., FLEET, C., TRITSARIS, K., DISSING, S., LEBOULCH, P. & CAO, Y. 2002. Placenta growth factor-1

antagonizes VEGF-induced angiogenesis and tumor growth by the formation of functionally inactive PlGF-1/VEGF heterodimers. *Cancer Cell,* 1**,** 99-108.

ESCUDERO-ESPARZA, A., MARTIN, T. A., DAVIES, M. L. & JIANG, W. G. 2009. PGF isoforms, PLGF-1 and PGF-2, in colorectal cancer and the prognostic significance. *Cancer Genomics Proteomics,* 6**,** 239-46.

ESPOSITO, K., NAPPO, F., MARFELLA, R., GIUGLIANO, G., GIUGLIANO, F., CIOTOLA, M., QUAGLIARO, L., CERIELLO, A. & GIUGLIANO, D. 2002. Inflammatory cytokine concentrations are acutely increased by hyperglycemia in humans: role of oxidative stress. *Circulation,* 106**,** 2067-72.

ESSERS, M. A., WEIJZEN, S., DE VRIES-SMITS, A. M., SAARLOOS, I., DE RUITER, N. D., BOS, J. L. & BURGERING, B. M. 2004. FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK. *Embo J,* 23**,** 4802-12.

FAILLA, C. M., ODORISIO, T., CIANFARANI, F., SCHIETROMA, C., PUDDU, P. & ZAMBRUNO, G. 2000. Placenta growth factor is induced in human keratinocytes during wound healing. *J Invest Dermatol,* 115**,** 388-95.

FANTIN, V. R., SPARLING, J. D., SLOT, J. W., KELLER, S. R., LIENHARD, G. E. & LAVAN, B. E. 1998. Characterization of insulin receptor substrate 4 in human embryonic kidney 293 cells. *J Biol Chem,* 273**,** 10726-32.

FANTIN, V. R., WANG, Q., LIENHARD, G. E. & KELLER, S. R. 2000. Mice lacking insulin receptor substrate 4 exhibit mild defects in growth, reproduction, and glucose homeostasis. *Am J Physiol Endocrinol Metab,* 278**,** E127-33.

FEDERICI, M., MENGHINI, R., MAURIELLO, A., HRIBAL, M. L., FERRELLI, F., LAURO, D., SBRACCIA, P., SPAGNOLI, L. G., SESTI, G. & LAURO, R. 2002. Insulin-dependent activation of endothelial nitric oxide synthase is impaired by O-linked glycosylation modification of signaling proteins in human coronary endothelial cells. *Circulation,* 106**,** 466-72.

FEENEY, S. A., SIMPSON, D. A., GARDINER, T. A., BOYLE, C., JAMISON, P. & STITT, A. W. 2003. Role of vascular endothelial growth factor and placental growth factors during retinal vascular development and hyaloid regression. *Invest Ophthalmol Vis Sci,* 44**,** 839-47.

FERNANDEZ-HERNANDO, C., ACKAH, E., YU, J., SUAREZ, Y., MURATA, T., IWAKIRI, Y., PRENDERGAST, J., MIAO, R. Q., BIRNBAUM, M. J. & SESSA, W. C. 2007. Loss of Akt1 leads to severe atherosclerosis and occlusive coronary artery disease. *Cell Metab,* 6**,** 446-57.

FERRARA, N. 2009. Vascular endothelial growth factor. *Arterioscler Thromb Vasc Biol,* 29**,** 789-91.

FERRARA, N., CARVER-MOORE, K., CHEN, H., DOWD, M., LU, L., O'SHEA, K. S., POWELL-BRAXTON, L., HILLAN, K. J. & MOORE, M. W. 1996. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature,* 380**,** 439-42.

FERRARA, N. & HENZEL, W. J. 1989. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem Biophys Res Commun,* 161**,** 851-8.

FIRE, A., XU, S., MONTGOMERY, M. K., KOSTAS, S. A., DRIVER, S. E. & MELLO, C. C. 1998. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature,* 391**,** 806-11.

FISCHER, C., JONCKX, B., MAZZONE, M., ZACCHIGNA, S., LOGES, S., PATTARINI, L., CHORIANOPOULOS, E., LIESENBORGHS, L., KOCH, M., DE MOL, M., AUTIERO, M., WYNS, S., PLAISANCE, S., MOONS, L., VAN ROOIJEN, N., GIACCA, M., STASSEN, J. M., DEWERCHIN, M., COLLEN, D. & CARMELIET, P. 2007. Anti-PlGF inhibits growth of VEGF(R)-inhibitor-resistant tumors without affecting healthy vessels. *Cell,* 131**,** 463-75.

FISCHER, C., MAZZONE, M., JONCKX, B. & CARMELIET, P. 2008. FLT1 and its ligands VEGFB and PlGF: drug targets for anti-angiogenic therapy? *Nat Rev Cancer,* 8**,** 942-56.

FLAMME, I. & RISAU, W. 1992. Induction of vasculogenesis and hematopoiesis in vitro. *Development,* 116**,** 435-9.

FOLKMAN, J. 1971. Tumor angiogenesis: therapeutic implications. *N Engl J Med,* 285**,** 1182-6.

FOLKMAN, J. 1995. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med,* 1**,** 27-31.

FOLKMAN, J. 1997. Angiogenesis and angiogenesis inhibition: an overview. *Exs,* 79**,** 1-8.

FOLKMAN, J., MERLER, E., ABERNATHY, C. & WILLIAMS, G. 1971. Isolation of a tumor factor responsible for angiogenesis. *J Exp Med,* 133**,** 275-88.

FOLLI, F., SAAD, M. J., BACKER, J. M. & KAHN, C. R. 1993. Regulation of phosphatidylinositol 3-kinase activity in liver and muscle of animal models of insulin-resistant and insulin-deficient diabetes mellitus. *J Clin Invest,* 92**,** 1787-94.

FONG, G. H., ROSSANT, J., GERTSENSTEIN, M. & BREITMAN, M. L. 1995. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature,* 376**,** 66-70.

FORD, E. S. 2005. Risks for all-cause mortality, cardiovascular disease, and diabetes associated with the metabolic syndrome: a summary of the evidence. *Diabetes Care,* 28**,** 1769-78.

FRAGOSO, R., IGREJA, C., CLODE, N., HENRIQUES, A., APPLETON, C., ZHU, Z., WU, Y. & DIAS, S. 2008. VEGF signaling on hematopoietic precursors restricts B-lymphoid commitment in vitro and in vivo. *Exp Hematol,* 36**,** 1329-1336.

FRANKE, T. F., KAPLAN, D. R., CANTLEY, L. C. & TOKER, A. 1997. Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. *Science,* 275**,** 665-8.

FRESCAS, D., VALENTI, L. & ACCILI, D. 2005. Nuclear trapping of the forkhead transcription factor FoxO1 via Sirt-dependent deacetylation promotes expression of glucogenetic genes. *J Biol Chem,* 280**,** 20589-95.

FUH, G., LI, B., CROWLEY, C., CUNNINGHAM, B. & WELLS, J. A. 1998. Requirements for binding and signaling of the kinase domain receptor for vascular endothelial growth factor. *J Biol Chem,* 273**,** 11197-204.

FUJIO, Y., GUO, K., MANO, T., MITSUUCHI, Y., TESTA, J. R. & WALSH, K. 1999. Cell cycle withdrawal promotes myogenic induction of Akt, a positive modulator of myocyte survival. *Mol Cell Biol,* 19**,** 5073-82.

FUJIO, Y. & WALSH, K. 1999. Akt mediates cytoprotection of endothelial cells by vascular endothelial growth factor in an anchorage-dependent manner. *J Biol Chem,* 274**,** 16349-54.

FUKUMURA, D., GOHONGI, T., KADAMBI, A., IZUMI, Y., ANG, J., YUN, C. O., BUERK, D. G., HUANG, P. L. & JAIN, R. K. 2001. Predominant role of endothelial nitric oxide synthase in vascular endothelial growth factor-induced angiogenesis and vascular permeability. *Proc Natl Acad Sci U S A,* 98**,** 2604-9.

FUKUOKA, M., DAITOKU, H., HATTA, M., MATSUZAKI, H., UMEMURA, S. & FUKAMIZU, A. 2003. Negative regulation of forkhead transcription factor AFX (Foxo4) by CBP-induced acetylation. *Int J Mol Med,* 12**,** 503-8.

FURUYAMA, T., KITAYAMA, K., SHIMODA, Y., OGAWA, M., SONE, K., YOSHIDA-ARAKI, K., HISATSUNE, H., NISHIKAWA, S., NAKAYAMA, K., NAKAYAMA, K., IKEDA, K., MOTOYAMA, N. & MORI, N. 2004. Abnormal angiogenesis in Foxo1 (Fkhr)-deficient mice. *J Biol Chem,* 279**,** 34741-9.

FURUYAMA, T., NAKAZAWA, T., NAKANO, I. & MORI, N. 2000. Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. *Biochem J,* 349**,** 629-34.

GABBAY, K. H. 1975. Hyperglycemia, polyol metabolism, and complications of diabetes mellitus. *Annu Rev Med,* 26**,** 521-36.

GALILI, N., DAVIS, R. J., FREDERICKS, W. J., MUKHOPADHYAY, S., RAUSCHER, F. J., 3RD, EMANUEL, B. S., ROVERA, G. & BARR, F. G. 1993. Fusion of a fork head domain gene to PAX3 in the solid tumour alveolar rhabdomyosarcoma. *Nat Genet,* 5**,** 230-5.

GAO, X., WANG, Z., MARTIN, J., ABUBUCKER, S., ZHANG, X., MITREVA, M. & HAWDON, J. M. 2010. Identification of hookworm DAF-16/FOXO response elements and direct gene targets. *PLoS One,* 5**,** e12289.

GARRIS, D. R. 1989. Morphometric analysis of obesity (ob/ob)- and diabetes (db/db)-associated hypothalamic neuronal degeneration in C57BL/KsJ mice. *Brain Res,* 501**,** 162-70.

GARRIS, D. R. & GARRIS, B. L. 2003. Diabetes (db/db) mutation-induced ovarian involution: progressive hypercytolipidemia. *Exp Biol Med (Maywood),* 228**,** 1040-50.

GEORGE, S., ROCHFORD, J. J., WOLFRUM, C., GRAY, S. L., SCHINNER, S., WILSON, J. C., SOOS, M. A., MURGATROYD, P. R., WILLIAMS, R. M., ACERINI, C. L., DUNGER, D. B., BARFORD, D., UMPLEBY, A. M., WAREHAM, N. J., DAVIES, H. A., SCHAFER, A. J., STOFFEL, M., O'RAHILLY, S. & BARROSO, I. 2004. A family with severe insulin resistance and diabetes due to a mutation in AKT2. *Science,* 304**,** 1325-8.

GERBER, H. P., MCMURTREY, A., KOWALSKI, J., YAN, M., KEYT, B. A., DIXIT, V. & FERRARA, N. 1998. Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *J Biol Chem,* 273**,** 30336-43.

GIARDINO, I., EDELSTEIN, D. & BROWNLEE, M. 1994. Nonenzymatic glycosylation in vitro and in bovine endothelial cells alters basic fibroblast growth factor activity. A model for intracellular glycosylation in diabetes. *J Clin Invest,* 94**,** 110-7.

GILMORE, T.D. (2006). Introduction to NF-kappaB: players, pathways, perspectives. Oncogene *25*, 6680-6684.

GOODYEAR, L. J., GIORGINO, F., SHERMAN, L. A., CAREY, J., SMITH, R. J. & DOHM, G. L. 1995. Insulin receptor phosphorylation, insulin receptor substrate-1 phosphorylation, and phosphatidylinositol 3-kinase activity are decreased in intact skeletal muscle strips from obese subjects. *J Clin Invest,* 95**,** 2195-204.

GRAHAM, F. L., SMILEY, J., RUSSELL, W. C. & NAIRN, R. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol,* 36**,** 59-74.

GRAVES, J. D., GOTOH, Y., DRAVES, K. E., AMBROSE, D., HAN, D. K., WRIGHT, M., CHERNOFF, J., CLARK, E. A. & KREBS, E. G. 1998. Caspase-mediated activation and induction of apoptosis by the mammalian Ste20-like kinase Mst1. *Embo J,* 17**,** 2224-34.

GREEN, C. J., LICHTLEN, P., HUYNH, N. T., YANOVSKY, M., LADEROUTE, K. R., SCHAFFNER, W. & MURPHY, B. J. 2001. Placenta growth factor gene expression is induced by hypoxia in fibroblasts: a central role for metal transcription factor-1. *Cancer Res,* 61**,** 2696-703.

GRUNDY, S. M., BREWER, H. B., JR., CLEEMAN, J. I., SMITH, S. C., JR. & LENFANT, C. 2004. Definition of metabolic syndrome: Report of the National Heart, Lung, and Blood Institute/American Heart Association conference on scientific issues related to definition. *Circulation,* 109**,** 433-8.

GUO, D., JIA, Q., SONG, H. Y., WARREN, R. S. & DONNER, D. B. 1995. Vascular endothelial cell growth factor promotes tyrosine phosphorylation of mediators of signal transduction that contain SH2 domains. Association with endothelial cell proliferation. *J Biol Chem,* 270**,** 6729-33.

GUZIK, T. J., MUSSA, S., GASTALDI, D., SADOWSKI, J., RATNATUNGA, C., PILLAI, R. & CHANNON, K. M. 2002. Mechanisms of increased vascular superoxide production in human diabetes mellitus: role of NAD(P)H oxidase and endothelial nitric oxide synthase. *Circulation,* 105**,** 1656-62.

HAGBERG, C. E., FALKEVALL, A., WANG, X., LARSSON, E., HUUSKO, J., NILSSON, I., VAN MEETEREN, L. A., SAMEN, E., LU, L., VANWILDEMEERSCH, M., KLAR, J., GENOVE, G., PIETRAS, K., STONE-ELANDER, S., CLAESSON-WELSH, L., YLA-HERTTUALA, S., LINDAHL, P. & ERIKSSON, U. 2010. Vascular endothelial growth factor B controls endothelial fatty acid uptake. *Nature,* 464**,** 917-21.

HASHIMOTO, N., KIDO, Y., UCHIDA, T., ASAHARA, S., SHIGEYAMA, Y., MATSUDA, T., TAKEDA, A., TSUCHIHASHI, D., NISHIZAWA, A., OGAWA, W., FUJIMOTO, Y., OKAMURA, H., ARDEN, K. C., HERRERA, P. L., NODA, T. & KASUGA, M. 2006. Ablation of PDK1 in pancreatic beta cells induces diabetes as a result of loss of beta cell mass. *Nat Genet,* 38**,** 589-93.

HATTORI, K., HEISSIG, B., WU, Y., DIAS, S., TEJADA, R., FERRIS, B., HICKLIN, D. J., ZHU, Z., BOHLEN, P., WITTE, L., HENDRIKX, J., HACKETT, N. R., CRYSTAL, R. G., MOORE, M. A., WERB, Z., LYDEN, D. & RAFII, S. 2002. Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1(+) stem cells from bone-marrow microenvironment. *Nat Med,* 8**,** 841-9.

HAWKINS, P. T., JACKSON, T. R. & STEPHENS, L. R. 1992. Platelet-derived growth factor stimulates synthesis of PtdIns(3,4,5)P3 by activating a PtdIns(4,5)P2 3-OH kinase. *Nature,* 358**,** 157-9.

HEISSIG, B., HATTORI, K., DIAS, S., FRIEDRICH, M., FERRIS, B., HACKETT, N. R., CRYSTAL, R. G., BESMER, P., LYDEN, D., MOORE, M. A., WERB, Z. & RAFII, S. 2002. Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. *Cell,* 109**,** 625-37.

HENDERSON, S. T. & JOHNSON, T. E. 2001. daf-16 integrates developmental and environmental inputs to mediate aging in the nematode Caenorhabditis elegans. *Curr Biol,* 11**,** 1975-80.

HEWETT, P. W. A., A 2006. *Angiogenesis in cervical neoplasia,* Oxford, UK, Blackwell publishing.

HEYMANS, S., LUTTUN, A., NUYENS, D., THEILMEIER, G., CREEMERS, E., MOONS, L., DYSPERSIN, G. D., CLEUTJENS, J. P., SHIPLEY, M., ANGELLILO, A., LEVI, M., NUBE, O., BAKER, A., KESHET, E., LUPU, F., HERBERT, J. M., SMITS, J. F., SHAPIRO, S. D., BAES, M., BORGERS, M., COLLEN, D., DAEMEN, M. J. & CARMELIET, P. 1999. Inhibition of plasminogen activators or matrix metalloproteinases prevents cardiac rupture but impairs therapeutic angiogenesis and causes cardiac failure. *Nat Med,* 5**,** 1135-42.

HIRATSUKA, S., MINOWA, O., KUNO, J., NODA, T. & SHIBUYA, M. 1998. Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. *Proc Natl Acad Sci U S A,* 95**,** 9349-54.

HIRSCHI, K. K. & D'AMORE, P. A. 1997. Control of angiogenesis by the pericyte: molecular mechanisms and significance. *Exs,* 79**,** 419-28.

HOEBEN, A., LANDUYT, B., HIGHLEY, M. S., WILDIERS, H., VAN OOSTEROM, A. T. & DE BRUIJN, E. A. 2004. Vascular endothelial growth factor and angiogenesis. *Pharmacol Rev,* 56**,** 549-80.

HOEKMAN, M. F., JACOBS, F. M., SMIDT, M. P. & BURBACH, J. P. 2006. Spatial and temporal expression of FoxO transcription factors in the developing and adult murine brain. *Gene Expr Patterns,* 6**,** 134-40.

HOSAKA, T., BIGGS, W. H., 3RD, TIEU, D., BOYER, A. D., VARKI, N. M., CAVENEE, W. K. & ARDEN, K. C. 2004. Disruption of forkhead transcription factor (FOXO) family members in mice reveals their functional diversification. *Proc Natl Acad Sci U S A,* 101**,** 2975-80.

HOUCK, K. A., FERRARA, N., WINER, J., CACHIANES, G., LI, B. & LEUNG, D. W. 1991. The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. *Mol Endocrinol,* 5**,** 1806-14.

HOUCK, K. A., LEUNG, D. W., ROWLAND, A. M., WINER, J. & FERRARA, N. 1992. Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. *J Biol Chem,* 267**,** 26031-7.

HRIBAL, M. L., NAKAE, J., KITAMURA, T., SHUTTER, J. R. & ACCILI, D. 2003. Regulation of insulin-like growth factor-dependent myoblast differentiation by Foxo forkhead transcription factors. *J Cell Biol,* 162**,** 535-41.

HU, M. C., LEE, D. F., XIA, W., GOLFMAN, L. S., OU-YANG, F., YANG, J. Y., ZOU, Y., BAO, S., HANADA, N., SASO, H., KOBAYASHI, R. & HUNG, M. C. 2004. IkappaB kinase promotes tumorigenesis through inhibition of forkhead FOXO3a. *Cell,* 117**,** 225-37.

HUANG, H., REGAN, K. M., LOU, Z., CHEN, J. & TINDALL, D. J. 2006. CDK2-dependent phosphorylation of FOXO1 as an apoptotic response to DNA damage. *Science,* 314**,** 294-7.

HUANG, J. & KONTOS, C. D. 2002. PTEN modulates vascular endothelial growth factor-mediated signaling and angiogenic effects. *J Biol Chem,* 277**,** 10760-6.

HUANG, K., ANDERSSON, C., ROOMANS, G. M., ITO, N. & CLAESSON-WELSH, L. 2001. Signaling properties of VEGF receptor-1 and -2 homo- and heterodimers. *Int J Biochem Cell Biol,* 33**,** 315-24.

IKEDA, M., HOSODA, Y., HIROSE, S., OKADA, Y. & IKEDA, E. 2000. Expression of vascular endothelial growth factor isoforms and their receptors Flt-1, KDR, and neuropilin-1 in synovial tissues of rheumatoid arthritis. *J Pathol,* 191**,** 426-33.

INGALLS, A. M., DICKIE, M. M. & SNELL, G. D. 1950. Obese, a new mutation in the house mouse. *J Hered,* 41**,** 317-8.

INOGUCHI, T., BATTAN, R., HANDLER, E., SPORTSMAN, J. R., HEATH, W. & KING, G. L. 1992. Preferential elevation of protein kinase C isoform beta II and diacylglycerol levels in the aorta and heart of diabetic rats: differential reversibility to glycemic control by islet cell transplantation. *Proc Natl Acad Sci U S A,* 89**,** 11059-63.

INOUE, M., ITOH, H., UEDA, M., NARUKO, T., KOJIMA, A., KOMATSU, R., DOI, K., OGAWA, Y., TAMURA, N., TAKAYA, K., IGAKI, T., YAMASHITA, J., CHUN, T. H., MASATSUGU, K., BECKER, A. E. & NAKAO, K. 1998. Vascular endothelial growth factor (VEGF) expression in human coronary atherosclerotic lesions: possible pathophysiological significance of VEGF in progression of atherosclerosis. *Circulation,* 98**,** 2108-16.

ISHIDA, A., MURRAY, J., SAITO, Y., KANTHOU, C., BENZAKOUR, O., SHIBUYA, M. & WIJELATH, E. S. 2001. Expression of vascular endothelial growth factor receptors in smooth muscle cells. *J Cell Physiol,* 188**,** 359-68.

ISHII, H., JIROUSEK, M. R., KOYA, D., TAKAGI, C., XIA, P., CLERMONT, A., BURSELL, S. E., KERN, T. S., BALLAS, L. M., HEATH, W. F., STRAMM, L. E., FEENER, E. P. & KING, G. L. 1996. Amelioration of vascular dysfunctions in diabetic rats by an oral PKC beta inhibitor. *Science,* 272**,** 728-31.

ITO, N., HUANG, K. & CLAESSON-WELSH, L. 2001. Signal transduction by VEGF receptor-1 wild type and mutant proteins. *Cell Signal,* 13**,** 849-54.

ITO, Y., AZROLAN, N., O'CONNELL, A., WALSH, A. & BRESLOW, J. L. 1990. Hypertriglyceridemia as a result of human apo CIII gene expression in transgenic mice. *Science,* 249**,** 790-3.

IVAN, M., KONDO, K., YANG, H., KIM, W., VALIANDO, J., OHH, M., SALIC, A., ASARA, J. M., LANE, W. S. & KAELIN, W. G., JR. 2001. HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. *Science,* 292**,** 464-8.

IWAMA, H., UEMURA, S., NAYA, N., IMAGAWA, K., TAKEMOTO, Y., ASAI, O., ONOUE, K., OKAYAMA, S., SOMEKAWA, S., KIDA, Y., TAKEDA, Y., NAKATANI, K., TAKAOKA, M., KAWATA, H., HORII, M., NAKAJIMA, T., DOI, N. & SAITO, Y. 2006. Cardiac expression of placental growth factor predicts the improvement of chronic phase left ventricular function in patients with acute myocardial infarction. *J Am Coll Cardiol,* 47**,** 1559-67.

IYER, S. & ACHARYA, K. R. 2002. Role of placenta growth factor in cardiovascular health. *Trends Cardiovasc Med,* 12**,** 128-34.

IYER, S., LEONIDAS, D. D., SWAMINATHAN, G. J., MAGLIONE, D., BATTISTI, M., TUCCI, M., PERSICO, M. G. & ACHARYA, K. R. 2001. The crystal structure of human placenta growth factor-1 (PlGF-1), an angiogenic protein, at 2.0 A resolution. *J Biol Chem,* 276**,** 12153-61.

JAAKKOLA, P., MOLE, D. R., TIAN, Y. M., WILSON, M. I., GIELBERT, J., GASKELL, S. J., KRIEGSHEIM, A., HEBESTREIT, H. F., MUKHERJI, M., SCHOFIELD, C. J., MAXWELL, P. H., PUGH, C. W. & RATCLIFFE, P. J. 2001. Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. *Science,* 292**,** 468-72.

JACKSON, M. W., ROBERTS, J. S., HECKFORD, S. E., RICCIARDELLI, C., STAHL, J., CHOONG, C., HORSFALL, D. J. & TILLEY, W. D. 2002. A potential autocrine role for vascular endothelial growth factor in prostate cancer. *Cancer Res,* 62**,** 854-9.

JACOBS, F. M., VAN DER HEIDE, L. P., WIJCHERS, P. J., BURBACH, J. P., HOEKMAN, M. F. & SMIDT, M. P. 2003. FoxO6, a novel member of the FoxO class of transcription factors with distinct shuttling dynamics. *J Biol Chem,* 278**,** 35959-67.

JAFFE, E. A., NACHMAN, R. L., BECKER, C. G. & MINICK, C. R. 1973. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J Clin Invest,* 52**,** 2745-56.

JAKEMAN, L. B., ARMANINI, M., PHILLIPS, H. S. & FERRARA, N. 1993. Developmental expression of binding sites and messenger ribonucleic acid for vascular endothelial growth factor suggests a role for this protein in vasculogenesis and angiogenesis. *Endocrinology,* 133**,** 848-59.

JAKEMAN, L. B., WINER, J., BENNETT, G. L., ALTAR, C. A. & FERRARA, N. 1992. Binding sites for vascular endothelial growth factor are localized on endothelial cells in adult rat tissues. *J Clin Invest,* 89**,** 244-53.

JAMES, D. E., BROWN, R., NAVARRO, J. & PILCH, P. F. 1988. Insulin-regulatable tissues express a unique insulin-sensitive glucose transport protein. *Nature,* 333**,** 183-5.

JAMES, D. E., STRUBE, M. & MUECKLER, M. 1989. Molecular cloning and characterization of an insulin-regulatable glucose transporter. *Nature,* 338**,** 83-7.

JIALAL, I., CRETTAZ, M., HACHIYA, H. L., KAHN, C. R., MOSES, A. C., BUZNEY, S. M. & KING, G. L. 1985. Characterization of the receptors for insulin and the insulin-like growth factors on micro- and macrovascular tissues. *Endocrinology,* 117**,** 1222-9.

JIANG, Z., PORE, N., CERNIGLIA, G. J., MICK, R., GEORGESCU, M. M., BERNHARD, E. J., HAHN, S. M., GUPTA, A. K. & MAITY, A. 2007. Phosphatase and tensin homologue deficiency in glioblastoma confers resistance to radiation and temozolomide that is reversed by the protease inhibitor nelfinavir. *Cancer Res,* 67**,** 4467-73.

JIN, C., MARSDEN, I., CHEN, X. & LIAO, X. 1998. Sequence specific collective motions in a winged helix DNA binding domain detected by 15N relaxation NMR. *Biochemistry,* 37**,** 6179-87.

JING, E., GESTA, S. & KAHN, C. R. 2007. SIRT2 regulates adipocyte differentiation through FoxO1 acetylation/deacetylation. *Cell Metab,* 6**,** 105-14.

JINNIN, M., MEDICI, D., PARK, L., LIMAYE, N., LIU, Y., BOSCOLO, E., BISCHOFF, J., VIKKULA, M., BOYE, E. & OLSEN, B. R. 2008. Suppressed NFAT-dependent VEGFR1 expression and constitutive VEGFR2 signaling in infantile hemangioma. *Nat Med,* 14**,** 1236-46.

JOUKOV, V., PAJUSOLA, K., KAIPAINEN, A., CHILOV, D., LAHTINEN, I., KUKK, E., SAKSELA, O., KALKKINEN, N. & ALITALO, K. 1996. A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *Embo J,* 15**,** 290-98.

KAESTNER, K. H., KNOCHEL, W. & MARTINEZ, D. E. 2000. Unified nomenclature for the winged helix/forkhead transcription factors. *Genes Dev,* 14**,** 142-6.

KAIPAINEN, A., KORHONEN, J., PAJUSOLA, K., APRELIKOVA, O., PERSICO, M. G., TERMAN, B. I. & ALITALO, K. 1993. The related FLT4, FLT1, and KDR receptor tyrosine kinases show distinct expression patterns in human fetal endothelial cells. *J Exp Med,* 178**,** 2077-88.

KAMEI, Y., MIZUKAMI, J., MIURA, S., SUZUKI, M., TAKAHASHI, N., KAWADA, T., TANIGUCHI, T. & EZAKI, O. 2003. A forkhead transcription factor FKHR up-regulates lipoprotein lipase expression in skeletal muscle. *FEBS Lett,* 536**,** 232-6.

KAMI, J., MURANAKA, K., YANAGI, Y., OBATA, R., TAMAKI, Y. & SHIBUYA, M. 2008. Inhibition of choroidal neovascularization by blocking vascular endothelial growth factor receptor tyrosine kinase. *Jpn J Ophthalmol,* 52**,** 91-8.

KAPLAN, R. N., RIBA, R. D., ZACHAROULIS, S., BRAMLEY, A. H., VINCENT, L., COSTA, C., MACDONALD, D. D., JIN, D. K., SHIDO, K., KERNS, S. A., ZHU, Z., HICKLIN, D., WU, Y., PORT, J. L., ALTORKI, N., PORT, E. R., RUGGERO, D., SHMELKOV, S. V., JENSEN, K. K., RAFII, S. & LYDEN, D. 2005. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature,* 438**,** 820-7.

KARKKAINEN, M. J., MAKINEN, T. & ALITALO, K. 2002. Lymphatic endothelium: a new frontier of metastasis research. *Nat Cell Biol,* 4**,** E2-5.

KATO, H., YOSHIKAWA, M., MIYAZAKI, T., NAKAJIMA, M., FUKAI, Y., MASUDA, N., FUKUCHI, M., MANDA, R., TSUKADA, K. & KUWANO, H. 2002. Expression of vascular endothelial growth factor (VEGF) and its receptors (Flt-1 and Flk-1) in esophageal squamous cell carcinoma. *Anticancer Res,* 22**,** 3977-84.

KATOH, O., TAUCHI, H., KAWAISHI, K., KIMURA, A. & SATOW, Y. 1995. Expression of the vascular endothelial growth factor (VEGF) receptor gene, KDR, in hematopoietic cells and inhibitory effect of VEGF on apoptotic cell death caused by ionizing radiation. *Cancer Res,* 55**,** 5687-92.

KECK, P. J., HAUSER, S. D., KRIVI, G., SANZO, K., WARREN, T., FEDER, J. & CONNOLLY, D. T. 1989. Vascular permeability factor, an endothelial cell mitogen related to PDGF. *Science,* 246**,** 1309-12.

KENDALL, R. L., WANG, G. & THOMAS, K. A. 1996. Identification of a natural soluble form of the vascular endothelial growth factor receptor, FLT-1, and its heterodimerization with KDR. *Biochem Biophys Res Commun,* 226**,** 324-8.

KEROUZ, N. J., HORSCH, D., PONS, S. & KAHN, C. R. 1997. Differential regulation of insulin receptor substrates-1 and -2 (IRS-1 and IRS-2) and phosphatidylinositol 3-kinase isoforms in liver and muscle of the obese diabetic (ob/ob) mouse. *J Clin Invest,* 100**,** 3164-72.

KHALIQ, A., FOREMAN, D., AHMED, A., WEICH, H., GREGOR, Z., MCLEOD, D. & BOULTON, M. 1998. Increased expression of placenta growth factor in proliferative diabetic retinopathy. *Lab Invest,* 78**,** 109-16.

KHALIQ, A., LI, X. F., SHAMS, M., SISI, P., ACEVEDO, C. A., WHITTLE, M. J., WEICH, H. & AHMED, A. 1996. Localisation of placenta growth factor (PIGF) in human term placenta. *Growth Factors,* 13**,** 243-50,color plates I-II,pre bk cov.

KHURANA, R., MOONS, L., SHAFI, S., LUTTUN, A., COLLEN, D., MARTIN, J. F., CARMELIET, P. & ZACHARY, I. C. 2005. Placental growth factor promotes atherosclerotic intimal thickening and macrophage accumulation. *Circulation,* 111**,** 2828-36.

KIM, H. S., SKURK, C., MAATZ, H., SHIOJIMA, I., IVASHCHENKO, Y., YOON, S. W., PARK, Y. B. & WALSH, K. 2005. Akt/FOXO3a signaling modulates the endothelial stress response through regulation of heat shock protein 70 expression. *Faseb J,* 19**,** 1042-4.

KIM, J. A., MONTAGNANI, M., KOH, K. K. & QUON, M. J. 2006. Reciprocal relationships between insulin resistance and endothelial dysfunction: molecular and pathophysiological mechanisms. *Circulation,* 113**,** 1888-904.

KITAMURA, T., NAKAE, J., KITAMURA, Y., KIDO, Y., BIGGS, W. H., 3RD, WRIGHT, C. V., WHITE, M. F., ARDEN, K. C. & ACCILI, D. 2002. The forkhead transcription factor Foxo1 links insulin signaling to Pdx1 regulation of pancreatic beta cell growth. *J Clin Invest,* 110**,** 1839-47.

KITAMURA, Y. I., KITAMURA, T., KRUSE, J. P., RAUM, J. C., STEIN, R., GU, W. & ACCILI, D. 2005. FoxO1 protects against pancreatic beta cell failure through NeuroD and MafA induction. *Cell Metab,* 2**,** 153-63.

KLAGSBRUN, M., TAKASHIMA, S. & MAMLUK, R. 2002. The role of neuropilin in vascular and tumor biology. *Adv Exp Med Biol,* 515**,** 33-48.

KLEYN, P. W., FAN, W., KOVATS, S. G., LEE, J. J., PULIDO, J. C., WU, Y., BERKEMEIER, L. R., MISUMI, D. J., HOLMGREN, L., CHARLAT, O., WOOLF, E. A., TAYBER, O., BRODY, T., SHU, P., HAWKINS, F., KENNEDY, B., BALDINI, L., EBELING, C., ALPERIN, G. D., DEEDS, J., LAKEY, N. D., CULPEPPER, J., CHEN, H., GLUCKSMANN-KUIS, M. A., CARLSON, G. A., DUYK, G. M. & MOORE, K. J. 1996. Identification and characterization of the mouse obesity gene tubby: a member of a novel gene family. *Cell,* 85**,** 281-90.

KNEDLER, A. & HAM, R. G. 1987. Optimized medium for clonal growth of human microvascular endothelial cells with minimal serum. *In Vitro Cell Dev Biol,* 23**,** 481-91.

KONDO, T. & KAHN, C. R. 2004. Altered insulin signaling in retinal tissue in diabetic states. *J Biol Chem,* 279**,** 37997-8006.

KOPS, G. J. & BURGERING, B. M. 1999. Forkhead transcription factors: new insights into protein kinase B (c-akt) signaling. *J Mol Med,* 77**,** 656-65.

KOPS, G. J., DANSEN, T. B., POLDERMAN, P. E., SAARLOOS, I., WIRTZ, K. W., COFFER, P. J., HUANG, T. T., BOS, J. L., MEDEMA, R. H. & BURGERING, B. M. 2002a. Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. *Nature,* 419**,** 316-21.

KOPS, G. J., DE RUITER, N. D., DE VRIES-SMITS, A. M., POWELL, D. R., BOS, J. L. & BURGERING, B. M. 1999. Direct control of the Forkhead transcription factor AFX by protein kinase B. *Nature,* 398**,** 630-4.

KOPS, G. J., MEDEMA, R. H., GLASSFORD, J., ESSERS, M. A., DIJKERS, P. F., COFFER, P. J., LAM, E. W. & BURGERING, B. M. 2002b. Control of cell cycle exit and entry by protein kinase B-regulated forkhead transcription factors. *Mol Cell Biol,* 22**,** 2025-36.

KUBOTA, N., TOBE, K., TERAUCHI, Y., ETO, K., YAMAUCHI, T., SUZUKI, R., TSUBAMOTO, Y., KOMEDA, K., NAKANO, R., MIKI, H., SATOH, S., SEKIHARA, H., SCIACCHITANO, S., LESNIAK, M., AIZAWA, S., NAGAI, R., KIMURA, S., AKANUMA, Y., TAYLOR, S. I. & KADOWAKI, T. 2000. Disruption of insulin receptor substrate 2 causes type 2 diabetes because of liver insulin resistance and lack of compensatory beta-cell hyperplasia. *Diabetes,* 49**,** 1880-9.

KWON, H. S., HUANG, B., UNTERMAN, T. G. & HARRIS, R. A. 2004. Protein kinase B-alpha inhibits human pyruvate dehydrogenase kinase-4 gene induction by dexamethasone through inactivation of FOXO transcription factors. *Diabetes,* 53**,** 899-910.

LAVAN, B. E., LANE, W. S. & LIENHARD, G. E. 1997. The 60-kDa phosphotyrosine protein in insulin-treated adipocytes is a new member of the insulin receptor substrate family. *J Biol Chem,* 272**,** 11439-43.

LEE, J., GRAY, A., YUAN, J., LUOH, S. M., AVRAHAM, H. & WOOD, W. I. 1996. Vascular endothelial growth factor-related protein: a ligand and specific activator of the tyrosine kinase receptor Flt4. *Proc Natl Acad Sci U S A,* 93**,** 1988-92.

LEE, S., CHEN, T. T., BARBER, C. L., JORDAN, M. C., MURDOCK, J., DESAI, S., FERRARA, N., NAGY, A., ROOS, K. P. & IRUELA-ARISPE, M. L. 2007. Autocrine VEGF signaling is required for vascular homeostasis. *Cell,* 130**,** 691-703.

LEHTINEN, M. K., YUAN, Z., BOAG, P. R., YANG, Y., VILLEN, J., BECKER, E. B., DIBACCO, S., DE LA IGLESIA, N., GYGI, S., BLACKWELL, T. K. & BONNI, A. 2006. A conserved MST-FOXO signaling pathway mediates oxidative-stress responses and extends life span. *Cell,* 125**,** 987-1001.

LEIBEL, R. L., CHUNG, W. K. & CHUA, S. C., JR. 1997. The molecular genetics of rodent single gene obesities. *J Biol Chem,* 272**,** 31937-40.

LEROITH, D., WERNER, H., BEITNER-JOHNSON, D. & ROBERTS, C. T., JR. 1995. Molecular and cellular aspects of the insulin-like growth factor I receptor. *Endocr Rev,* 16**,** 143-63.

LEVINE, R. J., LAM, C., QIAN, C., YU, K. F., MAYNARD, S. E., SACHS, B. P., SIBAI, B. M., EPSTEIN, F. H., ROMERO, R., THADHANI, R. & KARUMANCHI, S. A. 2006. Soluble endoglin and other circulating antiangiogenic factors in preeclampsia. *N Engl J Med,* 355**,** 992-1005.

LI, G., BARRETT, E. J., WANG, H., CHAI, W. & LIU, Z. 2005. Insulin at physiological concentrations selectively activates insulin but not insulin-like growth factor I (IGF-I) or insulin/IGF-I hybrid receptors in endothelial cells. *Endocrinology,* 146**,** 4690-6.

LI, J., YEN, C., LIAW, D., PODSYPANINA, K., BOSE, S., WANG, S. I., PUC, J., MILIARESIS, C., RODGERS, L., MCCOMBIE, R., BIGNER, S. H., GIOVANELLA, B. C., ITTMANN, M., TYCKO, B., HIBSHOOSH, H., WIGLER, M. H. & PARSONS, R. 1997. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science,* 275**,** 1943-7.

LI, Y. M., MITSUHASHI, T., WOJCIECHOWICZ, D., SHIMIZU, N., LI, J., STITT, A., HE, C., BANERJEE, D. & VLASSARA, H. 1996. Molecular identity and cellular distribution of advanced glycation endproduct receptors: relationship of p60 to OST-48 and p90 to 80K-H membrane proteins. *Proc Natl Acad Sci U S A,* 93**,** 11047-52.

LIETZKE, S. E., BOSE, S., CRONIN, T., KLARLUND, J., CHAWLA, A., CZECH, M. P. & LAMBRIGHT, D. G. 2000. Structural basis of 3-phosphoinositide recognition by pleckstrin homology domains. *Mol Cell,* 6**,** 385-94.

LIJNEN, H. R., CHRISTIAENS, V., SCROYEN, I., VOROS, G., TJWA, M., CARMELIET, P. & COLLEN, D. 2006. Impaired adipose tissue development in mice with inactivation of placental growth factor function. *Diabetes,* 55**,** 2698-704.

LIN, J., WU, H., TARR, P. T., ZHANG, C. Y., WU, Z., BOSS, O., MICHAEL, L. F., PUIGSERVER, P., ISOTANI, E., OLSON, E. N., LOWELL, B. B., BASSEL-DUBY, R. & SPIEGELMAN, B. M. 2002. Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. *Nature,* 418**,** 797-801.

LINDSTROM, P. 2007. The physiology of obese-hyperglycemic mice [ob/ob mice]. *ScientificWorldJournal,* 7**,** 666-85.

LIU, Y., COX, S. R., MORITA, T. & KOUREMBANAS, S. 1995. Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells. Identification of a 5' enhancer. *Circ Res,* 77**,** 638-43.

LONSER, R. R., GLENN, G. M., WALTHER, M., CHEW, E. Y., LIBUTTI, S. K., LINEHAN, W. M. & OLDFIELD, E. H. 2003. von Hippel-Lindau disease. *Lancet,* 361**,** 2059-67.

LORENZI, M., CAGLIERO, E. & TOLEDO, S. 1985. Glucose toxicity for human endothelial cells in culture. Delayed replication, disturbed cell cycle, and accelerated death. *Diabetes,* 34**,** 621-7.

LORENZO, C., OKOLOISE, M., WILLIAMS, K., STERN, M. P. & HAFFNER, S. M. 2003. The metabolic syndrome as predictor of type 2 diabetes: the San Antonio heart study. *Diabetes Care,* 26**,** 3153-9.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. 1951. Protein measurement with the Folin phenol reagent. *J Biol Chem,* 193**,** 265-75.

LUTTUN, A., TJWA, M., MOONS, L., WU, Y., ANGELILLO-SCHERRER, A., LIAO, F., NAGY, J. A., HOOPER, A., PRILLER, J., DE KLERCK, B., COMPERNOLLE, V., DACI, E., BOHLEN, P., DEWERCHIN, M., HERBERT, J. M., FAVA, R., MATTHYS, P., CARMELIET, G., COLLEN, D., DVORAK, H. F., HICKLIN, D. J. & CARMELIET, P. 2002. Revascularization

of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. *Nat Med,* 8**,** 831-40.

LYSS, G., KNORRE, A., SCHMIDT, T. J., PAHL, H. L. & MERFORT, I. 1998. The anti-inflammatory sesquiterpene lactone helenalin inhibits the transcription factor NF-kappaB by directly targeting p65. *J Biol Chem,* 273**,** 33508-16.

LYTTLE, D. J., FRASER, K. M., FLEMING, S. B., MERCER, A. A. & ROBINSON, A. J. 1994. Homologs of vascular endothelial growth factor are encoded by the poxvirus orf virus. *J Virol,* 68**,** 84-92.

MADAMANCHI, N. R., VENDROV, A. & RUNGE, M. S. 2005. Oxidative stress and vascular disease. *Arterioscler Thromb Vasc Biol,* 25**,** 29-38.

MAEHAMA, T. & DIXON, J. E. 1999. PTEN: a tumour suppressor that functions as a phospholipid phosphatase. *Trends Cell Biol,* 9**,** 125-8.

MAGLIONE, D., GUERRIERO, V., RAMBALDI, M., RUSSO, G. & PERSICO, M. G. 1993. Translation of the placenta growth factor mRNA is severely affected by a small open reading frame localized in the 5' untranslated region. *Growth Factors,* 8**,** 141-52.

MAGLIONE, D., GUERRIERO, V., VIGLIETTO, G., DELLI-BOVI, P. & PERSICO, M. G. 1991. Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor. *Proc Natl Acad Sci U S A,* 88**,** 9267-71.

MAIESE, K., CHONG, Z. Z., SHANG, Y. C. & HOU, J. 2009. FoxO proteins: cunning concepts and considerations for the cardiovascular system. *Clin Sci (Lond),* 116**,** 191-203.

MALARSTIG, A., TENNO, T., JOSSAN, S., ABERG, M. & SIEGBAHN, A. 2003. A quantitative real-time PCR method for tissue factor mRNA. *Thromb Res,* 112**,** 175-83.

MANNING, B. D. & CANTLEY, L. C. 2007. AKT/PKB signaling: navigating downstream. *Cell,* 129**,** 1261-74.

MARCHETTI, V., MENGHINI, R., RIZZA, S., VIVANTI, A., FECCIA, T., LAURO, D., FUKAMIZU, A., LAURO, R. & FEDERICI, M. 2006. Benfotiamine counteracts glucose toxicity effects on endothelial progenitor cell differentiation via Akt/FoxO signaling. *Diabetes,* 55**,** 2231-7.

MARTINEZ-GAC, L., MARQUES, M., GARCIA, Z., CAMPANERO, M. R. & CARRERA, A. C. 2004. Control of cyclin G2 mRNA expression by forkhead transcription factors: novel mechanism for cell cycle control by phosphoinositide 3-kinase and forkhead. *Mol Cell Biol,* 24**,** 2181-9.

MATSUMOTO, K., SUZUKI, K., KOIKE, H., HASUMI, M., MATSUI, H., OKUGI, H., SHIBATA, Y., ITO, K. & YAMANAKA, H. 2003a. Placental growth factor gene expression in human prostate cancer and benign prostate hyperplasia. *Anticancer Res,* 23**,** 3767-73.

MATSUMOTO, K., SUZUKI, K., KOIKE, H., OKAMURA, K., TSUCHIYA, K., UCHIDA, T., TAKEZAWA, Y., KOBAYASHI, M. & YAMANAKA, H. 2003b. Prognostic significance of plasma placental growth factor levels in renal cell cancer: an association with clinical characteristics and vascular endothelial growth factor levels. *Anticancer Res,* 23**,** 4953-8.

MATSUMOTO, M., HAN, S., KITAMURA, T. & ACCILI, D. 2006. Dual role of transcription factor FoxO1 in controlling hepatic insulin sensitivity and lipid metabolism. *J Clin Invest,* 116**,** 2464-72.

MATTHEWS, W., JORDAN, C. T., GAVIN, M., JENKINS, N. A., COPELAND, N. G. & LEMISCHKA, I. R. 1991. A receptor tyrosine kinase cDNA isolated from a population of enriched primitive hematopoietic cells and exhibiting close genetic linkage to c-kit. *Proc Natl Acad Sci U S A,* 88**,** 9026-30.

MAYNARD, S. E., MIN, J. Y., MERCHAN, J., LIM, K. H., LI, J., MONDAL, S., LIBERMANN, T. A., MORGAN, J. P., SELLKE, F. W., STILLMAN, I. E., EPSTEIN, F. H., SUKHATME, V. P. & KARUMANCHI, S. A. 2003. Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *J Clin Invest,* 111**,** 649-58.

MCLELLAN, A. C., THORNALLEY, P. J., BENN, J. & SONKSEN, P. H. 1994. Glyoxalase system in clinical diabetes mellitus and correlation with diabetic complications. *Clin Sci (Lond),* 87**,** 21-9.

MEDEMA, R. H., KOPS, G. J., BOS, J. L. & BURGERING, B. M. 2000. AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. *Nature,* 404**,** 782-7.

MEISTER, G. & TUSCHL, T. 2004. Mechanisms of gene silencing by double-stranded RNA. *Nature,* 431**,** 343-9.

MICHAEL, M. D., KULKARNI, R. N., POSTIC, C., PREVIS, S. F., SHULMAN, G. I., MAGNUSON, M. A. & KAHN, C. R. 2000. Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction. *Mol Cell,* 6**,** 87-97.

MIGDAL, M., HUPPERTZ, B., TESSLER, S., COMFORTI, A., SHIBUYA, M., REICH, R., BAUMANN, H. & NEUFELD, G. 1998. Neuropilin-1 is a placenta growth factor-2 receptor. *J Biol Chem,* 273**,** 22272-8.

MIKI, H., YAMAUCHI, T., SUZUKI, R., KOMEDA, K., TSUCHIDA, A., KUBOTA, N., TERAUCHI, Y., KAMON, J., KABURAGI, Y., MATSUI, J., AKANUMA, Y., NAGAI, R., KIMURA, S., TOBE, K. & KADOWAKI, T. 2001. Essential role of insulin receptor substrate 1 (IRS-1) and IRS-2 in adipocyte differentiation. *Mol Cell Biol,* 21**,** 2521-32.

MIN, J. K., KIM, Y. M., KIM, S. W., KWON, M. C., KONG, Y. Y., HWANG, I. K., WON, M. H., RHO, J. & KWON, Y. G. 2005. TNF-related activation-induced cytokine enhances leukocyte adhesiveness: induction of ICAM-1 and VCAM-1 via TNF receptor-associated factor and protein kinase C-dependent NF-kappaB activation in endothelial cells. *J Immunol,* 175**,** 531-40.

MIQUEROL, L., LANGILLE, B. L. & NAGY, A. 2000. Embryonic development is disrupted by modest increases in vascular endothelial growth factor gene expression. *Development,* 127**,** 3941-6.

MITAMURA, Y., TASHIMO, A., NAKAMURA, Y., TAGAWA, H., OHTSUKA, K., MIZUE, Y. & NISHIHIRA, J. 2002. Vitreous levels of placenta growth factor and vascular endothelial growth factor in patients with proliferative diabetic retinopathy. *Diabetes Care,* 25**,** 2352.

MIYAZAKI, T., KATAGIRI, H., KANEGAE, Y., TAKAYANAGI, H., SAWADA, Y., YAMAMOTO, A., PANDO, M. P., ASANO, T., VERMA, I. M., ODA, H., NAKAMURA, K. & TANAKA, S.

2000. Reciprocal role of ERK and NF-kappaB pathways in survival and activation of osteoclasts. *J Cell Biol,* 148**,** 333-42.

MOHAMED, Q., GILLIES, M. C. & WONG, T. Y. 2007. Management of diabetic retinopathy: a systematic review. *Jama,* 298**,** 902-16.

MONTAGNANI, M., GOLOVCHENKO, I., KIM, I., KOH, G. Y., GOALSTONE, M. L., MUNDHEKAR, A. N., JOHANSEN, M., KUCIK, D. F., QUON, M. J. & DRAZNIN, B. 2002. Inhibition of phosphatidylinositol 3-kinase enhances mitogenic actions of insulin in endothelial cells. *J Biol Chem,* 277**,** 1794-9.

MORBIDELLI, L., CHANG, C. H., DOUGLAS, J. G., GRANGER, H. J., LEDDA, F. & ZICHE, M. 1996. Nitric oxide mediates mitogenic effect of VEGF on coronary venular endothelium. *Am J Physiol,* 270**,** H411-5.

MORIGI, M., ANGIOLETTI, S., IMBERTI, B., DONADELLI, R., MICHELETTI, G., FIGLIUZZI, M., REMUZZI, A., ZOJA, C. & REMUZZI, G. 1998. Leukocyte-endothelial interaction is augmented by high glucose concentrations and hyperglycemia in a NF-kB-dependent fashion. *J Clin Invest,* 101**,** 1905-15.

MOTTA, M. C., DIVECHA, N., LEMIEUX, M., KAMEL, C., CHEN, D., GU, W., BULTSMA, Y., MCBURNEY, M. & GUARENTE, L. 2004. Mammalian SIRT1 represses forkhead transcription factors. *Cell,* 116**,** 551-63.

MUKAI, Y., RIKITAKE, Y., SHIOJIMA, I., WOLFRUM, S., SATOH, M., TAKESHITA, K., HIROI, Y., SALOMONE, S., KIM, H. H., BENJAMIN, L. E., WALSH, K. & LIAO, J. K. 2006. Decreased vascular lesion formation in mice with inducible endothelial-specific expression of protein kinase Akt. *J Clin Invest,* 116**,** 334-43.

MULLER, Y. A., LI, B., CHRISTINGER, H. W., WELLS, J. A., CUNNINGHAM, B. C. & DE VOS, A. M. 1997. Vascular endothelial growth factor: crystal structure and functional mapping of the kinase domain receptor binding site. *Proc Natl Acad Sci U S A,* 94**,** 7192-7.

MURAKAMI, M., IWAI, S., HIRATSUKA, S., YAMAUCHI, M., NAKAMURA, K., IWAKURA, Y. & SHIBUYA, M. 2006. Signaling of vascular endothelial growth factor receptor-1 tyrosine kinase promotes rheumatoid arthritis through activation of monocytes/macrophages. *Blood,* 108**,** 1849-56.

MYERS, M. G., JR., BACKER, J. M., SUN, X. J., SHOELSON, S., HU, P., SCHLESSINGER, J., YOAKIM, M., SCHAFFHAUSEN, B. & WHITE, M. F. 1992. IRS-1 activates phosphatidylinositol 3'-kinase by associating with src homology 2 domains of p85. *Proc Natl Acad Sci U S A,* 89**,** 10350-4.

MYERS, M. G., JR., MENDEZ, R., SHI, P., PIERCE, J. H., RHOADS, R. & WHITE, M. F. 1998. The COOH-terminal tyrosine phosphorylation sites on IRS-1 bind SHP-2 and negatively regulate insulin signaling. *J Biol Chem,* 273**,** 26908-14.

NAGGERT, J. K., FRICKER, L. D., VARLAMOV, O., NISHINA, P. M., ROUILLE, Y., STEINER, D. F., CARROLL, R. J., PAIGEN, B. J. & LEITER, E. H. 1995. Hyperproinsulinaemia in obese fat/fat mice associated with a carboxypeptidase E mutation which reduces enzyme activity. *Nat Genet,* 10**,** 135-42.

NAGY, J. A., VASILE, E., FENG, D., SUNDBERG, C., BROWN, L. F., DETMAR, M. J., LAWITTS, J. A., BENJAMIN, L., TAN, X., MANSEAU, E. J., DVORAK, A. M. & DVORAK, H. F. 2002. Vascular permeability factor/vascular endothelial growth factor induces lymphangiogenesis as well as angiogenesis. *J Exp Med,* 196**,** 1497-506.

NAKAE, J., KITAMURA, T., KITAMURA, Y., BIGGS, W. H., 3RD, ARDEN, K. C. & ACCILI, D. 2003. The forkhead transcription factor Foxo1 regulates adipocyte differentiation. *Dev Cell,* 4**,** 119-29.

NAKAE, J., KITAMURA, T., SILVER, D. L. & ACCILI, D. 2001. The forkhead transcription factor Foxo1 (Fkhr) confers insulin sensitivity onto glucose-6-phosphatase expression. *J Clin Invest,* 108**,** 1359-67.

NAKAMURA, N., RAMASWAMY, S., VAZQUEZ, F., SIGNORETTI, S., LODA, M. & SELLERS, W. R. 2000. Forkhead transcription factors are critical effectors of cell death and cell cycle arrest downstream of PTEN. *Mol Cell Biol,* 20**,** 8969-82.

NASRIN, N., OGG, S., CAHILL, C. M., BIGGS, W., NUI, S., DORE, J., CALVO, D., SHI, Y., RUVKUN, G. & ALEXANDER-BRIDGES, M. C. 2000. DAF-16 recruits the CREB-binding protein coactivator complex to the insulin-like growth factor binding protein 1 promoter in HepG2 cells. *Proc Natl Acad Sci U S A,* 97**,** 10412-7.

NATHAN, D. M., LACHIN, J., CLEARY, P., ORCHARD, T., BRILLON, D. J., BACKLUND, J. Y., O'LEARY, D. H. & GENUTH, S. 2003. Intensive diabetes therapy and carotid intima-media thickness in type 1 diabetes mellitus. *N Engl J Med,* 348**,** 2294-303.

NEUFELD, G., COHEN, T., SHRAGA, N., LANGE, T., KESSLER, O. & HERZOG, Y. 2002. The neuropilins: multifunctional semaphorin and VEGF receptors that modulate axon guidance and angiogenesis. *Trends Cardiovasc Med,* 12**,** 13-9.

NICOSIA, R. F., NICOSIA, S. V. & SMITH, M. 1994. Vascular endothelial growth factor, platelet-derived growth factor, and insulin-like growth factor-1 promote rat aortic angiogenesis in vitro. *Am J Pathol,* 145**,** 1023-9.

NISHIKAWA, T., EDELSTEIN, D., DU, X. L., YAMAGISHI, S., MATSUMURA, T., KANEDA, Y., YOREK, M. A., BEEBE, D., OATES, P. J., HAMMES, H. P., GIARDINO, I. & BROWNLEE, M. 2000. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature,* 404**,** 787-90.

NISHIMOTO, F., SAKATA, M., MINEKAWA, R., OKAMOTO, Y., MIYAKE, A., ISOBE, A., YAMAMOTO, T., TAKEDA, T., ISHIDA, E., SAWADA, K., MORISHIGE, K. & KIMURA, T. 2009. Metal transcription factor-1 is involved in hypoxia-dependent regulation of placenta growth factor in trophoblast-derived cells. *Endocrinology,* 150**,** 1801-8.

NITERT, M. D., CHISALITA, S. I., OLSSON, K., BORNFELDT, K. E. & ARNQVIST, H. J. 2005. IGF-I/insulin hybrid receptors in human endothelial cells. *Mol Cell Endocrinol,* 229**,** 31-7.

NYBERG-HOFFMAN, C. & AGUILAR-CORDOVA, E. 1999. Instability of adenoviral vectors during transport and its implication for clinical studies. *Nat Med,* 5**,** 955-7.

OBSIL, T. & OBSILOVA, V. 2008. Structure/function relationships underlying regulation of FOXO transcription factors. *Oncogene,* 27**,** 2263-75.

ODORISIO, T., CIANFARANI, F., FAILLA, C. M. & ZAMBRUNO, G. 2006. The placenta growth factor in skin angiogenesis. *J Dermatol Sci,* 41**,** 11-9.

OKANO, J., GASLIGHTWALA, I., BIRNBAUM, M. J., RUSTGI, A. K. & NAKAGAWA, H. 2000. Akt/protein kinase B isoforms are differentially regulated by epidermal growth factor stimulation. *J Biol Chem,* 275**,** 30934-42.

OLOFSSON, B., PAJUSOLA, K., KAIPAINEN, A., VON EULER, G., JOUKOV, V., SAKSELA, O., ORPANA, A., PETTERSSON, R. F., ALITALO, K. & ERIKSSON, U. 1996. Vascular endothelial growth factor B, a novel growth factor for endothelial cells. *Proc Natl Acad Sci U S A,* 93**,** 2576-81.

ORLANDINI, M., MARCONCINI, L., FERRUZZI, R. & OLIVIERO, S. 1996. Identification of a c-fos-induced gene that is related to the platelet-derived growth factor/vascular endothelial growth factor family. *Proc Natl Acad Sci U S A,* 93**,** 11675-80.

OVERDIER, D. G., PORCELLA, A. & COSTA, R. H. 1994. The DNA-binding specificity of the hepatocyte nuclear factor 3/forkhead domain is influenced by amino-acid residues adjacent to the recognition helix. *Mol Cell Biol,* 14**,** 2755-66.

PAIK, J. H., KOLLIPARA, R., CHU, G., JI, H., XIAO, Y., DING, Z., MIAO, L., TOTHOVA, Z., HORNER, J. W., CARRASCO, D. R., JIANG, S., GILLILAND, D. G., CHIN, L., WONG, W. H., CASTRILLON, D. H. & DEPINHO, R. A. 2007. FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. *Cell,* 128**,** 309-23.

PAJUSOLA, K., APRELIKOVA, O., KORHONEN, J., KAIPAINEN, A., PERTOVAARA, L., ALITALO, R. & ALITALO, K. 1992. FLT4 receptor tyrosine kinase contains seven immunoglobulin-like loops and is expressed in multiple human tissues and cell lines. *Cancer Res,* 52**,** 5738-43.

PARENTI, A., MORBIDELLI, L., CUI, X. L., DOUGLAS, J. G., HOOD, J. D., GRANGER, H. J., LEDDA, F. & ZICHE, M. 1998. Nitric oxide is an upstream signal of vascular endothelial growth factor-induced extracellular signal-regulated kinase1/2 activation in postcapillary endothelium. *J Biol Chem,* 273**,** 4220-6.

PARK, J. E., CHEN, H. H., WINER, J., HOUCK, K. A. & FERRARA, N. 1994. Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR. *J Biol Chem,* 269**,** 25646-54.

PARK, J. E., KELLER, G. A. & FERRARA, N. 1993. The vascular endothelial growth factor (VEGF) isoforms: differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. *Mol Biol Cell,* 4**,** 1317-26.

PARK, K. W., KIM, D. H., YOU, H. J., SIR, J. J., JEON, S. I., YOUN, S. W., YANG, H. M., SKURK, C., PARK, Y. B., WALSH, K. & KIM, H. S. 2005. Activated forkhead transcription factor inhibits neointimal hyperplasia after angioplasty through induction of p27. *Arterioscler Thromb Vasc Biol,* 25**,** 742-7.

PARR, C., WATKINS, G., BOULTON, M., CAI, J. & JIANG, W. G. 2005. Placenta growth factor is over-expressed and has prognostic value in human breast cancer. *Eur J Cancer,* 41**,** 2819-27.

PATZ, A. 1978. Current concepts in ophthalmology. Retinal vascular diseases. *N Engl J Med,* 298**,** 1451-4.

PELLEYMOUNTER, M. A., CULLEN, M. J., BAKER, M. B., HECHT, R., WINTERS, D., BOONE, T. & COLLINS, F. 1995. Effects of the obese gene product on body weight regulation in ob/ob mice. *Science,* 269**,** 540-3.

PEPPER, M. S., FERRARA, N., ORCI, L. & MONTESANO, R. 1992. Potent synergism between vascular endothelial growth factor and basic fibroblast growth factor in the induction of angiogenesis in vitro. *Biochem Biophys Res Commun,* 189**,** 824-31.

PEPPER, M. S., MANDRIOTA, S. J., JELTSCH, M., KUMAR, V. & ALITALO, K. 1998. Vascular endothelial growth factor (VEGF)-C synergizes with basic fibroblast growth factor and VEGF in the induction of angiogenesis in vitro and alters endothelial cell extracellular proteolytic activity. *J Cell Physiol,* 177**,** 439-52.

PERROT, V. & RECHLER, M. M. 2005. The coactivator p300 directly acetylates the forkhead transcription factor Foxo1 and stimulates Foxo1-induced transcription. *Mol Endocrinol,* 19**,** 2283-98.

PERSICO, M. G., VINCENTI, V. & DIPALMA, T. 1999. Structure, expression and receptor-binding properties of placenta growth factor (PlGF). *Curr Top Microbiol Immunol,* 237**,** 31-40.

PETERSEN, K. F. & SHULMAN, G. I. 2006. Etiology of insulin resistance. *Am J Med,* 119**,** S10-6.

PIERROU, S., HELLQVIST, M., SAMUELSSON, L., ENERBACK, S. & CARLSSON, P. 1994. Cloning and characterization of seven human forkhead proteins: binding site specificity and DNA bending. *Embo J,* 13**,** 5002-12.

PILARCZYK, K., SATTLER, K. J., GALILI, O., VERSARI, D., OLSON, M. L., MEYER, F. B., ZHU, X. Y., LERMAN, L. O. & LERMAN, A. 2008. Placenta growth factor expression in human atherosclerotic carotid plaques is related to plaque destabilization. *Atherosclerosis,* 196**,** 333-40.

PIPP, F., HEIL, M., ISSBRUCKER, K., ZIEGELHOEFFER, T., MARTIN, S., VAN DEN HEUVEL, J., WEICH, H., FERNANDEZ, B., GOLOMB, G., CARMELIET, P., SCHAPER, W. & CLAUSS, M. 2003. VEGFR-1-selective VEGF homologue PlGF is arteriogenic: evidence for a monocyte-mediated mechanism. *Circ Res,* 92**,** 378-85.

PLAS, D. R. & THOMPSON, C. B. 2003. Akt activation promotes degradation of tuberin and FOXO3a via the proteasome. *J Biol Chem,* 278**,** 12361-6.

PLATE, K. H., BREIER, G., WEICH, H. A., MENNEL, H. D. & RISAU, W. 1994. Vascular endothelial growth factor and glioma angiogenesis: coordinate induction of VEGF receptors, distribution of VEGF protein and possible in vivo regulatory mechanisms. *Int J Cancer,* 59**,** 520-9.

PLOUET, J. & MOUKADIRI, H. 1990. Specific binding of vasculotropin to bovine brain capillary endothelial cells. *Biochimie,* 72**,** 51-5.

POTENTE, M., URBICH, C., SASAKI, K., HOFMANN, W. K., HEESCHEN, C., AICHER, A., KOLLIPARA, R., DEPINHO, R. A., ZEIHER, A. M. & DIMMELER, S. 2005. Involvement of Foxo transcription factors in angiogenesis and postnatal neovascularization. *J Clin Invest,* 115**,** 2382-92.

POTENZA, M. A., ADDABBO, F. & MONTAGNANI, M. 2009a. Vascular actions of insulin with implications for endothelial dysfunction. *Am J Physiol Endocrinol Metab,* 297**,** E568-77.

POTENZA, M. A., GAGLIARDI, S., NACCI, C., CARRATU, M. R. & MONTAGNANI, M. 2009b. Endothelial dysfunction in diabetes: from mechanisms to therapeutic targets. *Curr Med Chem,* 16**,** 94-112.

POTENZA, M. A., MARASCIULO, F. L., CHIEPPA, D. M., BRIGIANI, G. S., FORMOSO, G., QUON, M. J. & MONTAGNANI, M. 2005. Insulin resistance in spontaneously hypertensive rats is associated with endothelial dysfunction characterized by imbalance between NO and ET-1 production. *Am J Physiol Heart Circ Physiol,* 289**,** H813-22.

PUIGSERVER, P., RHEE, J., DONOVAN, J., WALKEY, C. J., YOON, J. C., ORIENTE, F., KITAMURA, Y., ALTOMONTE, J., DONG, H., ACCILI, D. & SPIEGELMAN, B. M. 2003. Insulin-regulated hepatic gluconeogenesis through FOXO1-PGC-1alpha interaction. *Nature,* 423**,** 550-5.

QUAGLIARO, L., PICONI, L., ASSALONI, R., DA ROS, R., MAIER, A., ZUODAR, G. & CERIELLO, A. 2005. Intermittent high glucose enhances ICAM-1, VCAM-1 and E-selectin expression in human umbilical vein endothelial cells in culture: the distinct role of protein kinase C and mitochondrial superoxide production. *Atherosclerosis,* 183**,** 259-67.

QUAGLIARO, L., PICONI, L., ASSALONI, R., MARTINELLI, L., MOTZ, E. & CERIELLO, A. 2003. Intermittent high glucose enhances apoptosis related to oxidative stress in human umbilical vein endothelial cells: the role of protein kinase C and NAD(P)H-oxidase activation. *Diabetes,* 52**,** 2795-804.

RANDI, A. M., SPERONE, A., DRYDEN, N. H. & BIRDSEY, G. M. 2009. Regulation of angiogenesis by ETS transcription factors. *Biochem Soc Trans,* 37**,** 1248-53.

REAVEN, G. M. 2005. The insulin resistance syndrome: definition and dietary approaches to treatment. *Annu Rev Nutr,* 25**,** 391-406.

RISAU, W. 1995. Differentiation of endothelium. *Faseb J,* 9**,** 926-33.

RISAU, W. 1997. Mechanisms of angiogenesis. *Nature,* 386**,** 671-4.

ROLNY, C., MAZZONE, M., TUGUES, S., LAOUI, D., JOHANSSON, I., COULON, C., SQUADRITO, M. L., SEGURA, I., LI, X., KNEVELS, E., COSTA, S., VINCKIER, S., DRESSELAER, T., AKERUD, P., DE MOL, M., SALOMAKI, H., PHILLIPSON, M., WYNS, S., LARSSON, E., BUYSSCHAERT, I., BOTLING, J., HIMMELREICH, U., VAN GINDERACHTER, J. A., DE PALMA, M., DEWERCHIN, M., CLAESSON-WELSH, L. & CARMELIET, P. 2011. HRG inhibits tumor growth and metastasis by inducing macrophage polarization and vessel normalization through downregulation of PlGF. *Cancer Cell,* 19**,** 31-44.

ROSNET, O., MATTEI, M. G., MARCHETTO, S. & BIRNBAUM, D. 1991. Isolation and chromosomal localization of a novel FMS-like tyrosine kinase gene. *Genomics,* 9**,** 380-5.

ROSS, R. 1993. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature,* 362**,** 801-9.

ROSS, R. 1999. Atherosclerosis--an inflammatory disease. *N Engl J Med,* 340**,** 115-26.

SAFRAN, M. & KAELIN, W. G., JR. 2003. HIF hydroxylation and the mammalian oxygen-sensing pathway. *J Clin Invest,* 111**,** 779-83.

SALTIEL, A. R. & KAHN, C. R. 2001. Insulin signalling and the regulation of glucose and lipid metabolism. *Nature,* 414**,** 799-806.

SAMUEL, V. T., CHOI, C. S., PHILLIPS, T. G., ROMANELLI, A. J., GEISLER, J. G., BHANOT, S., MCKAY, R., MONIA, B., SHUTTER, J. R., LINDBERG, R. A., SHULMAN, G. I. & VENIANT, M. M. 2006. Targeting foxo1 in mice using antisense oligonucleotide improves hepatic and peripheral insulin action. *Diabetes,* 55**,** 2042-50.

SARBASSOV, D. D., GUERTIN, D. A., ALI, S. M. & SABATINI, D. M. 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science,* 307**,** 1098-101.

SARTORETTO, J. L., OLIVEIRA, M. A., NIGRO, D., CARVALHO, M. H., TOSTES, R. C. & FORTES, Z. B. 2007. Constrictor responses to noradrenaline, hemodynamic profile, and superoxide levels measured by hydroethidine oxidation in diabetic rats. *Biol Pharm Bull,* 30**,** 1938-42.

SATO, T. N., TOZAWA, Y., DEUTSCH, U., WOLBURG-BUCHHOLZ, K., FUJIWARA, Y., GENDRON-MAGUIRE, M., GRIDLEY, T., WOLBURG, H., RISAU, W. & QIN, Y. 1995. Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature,* 376**,** 70-4.

SATTAR, A., ROONEY, P., KUMAR, S., PYE, D., WEST, D. C., SCOTT, I. & LEDGER, P. 1994. Application of angiogenic oligosaccharides of hyaluronan increases blood vessel numbers in rat skin. *J Invest Dermatol,* 103**,** 576-9.

SAWANO, A., TAKAHASHI, T., YAMAGUCHI, S. & SHIBUYA, M. 1997. The phosphorylated 1169-tyrosine containing region of flt-1 kinase (VEGFR-1) is a major binding site for PLCgamma. *Biochem Biophys Res Commun,* 238**,** 487-91.

SCHALKWIJK, C. G. & STEHOUWER, C. D. 2005. Vascular complications in diabetes mellitus: the role of endothelial dysfunction. *Clin Sci (Lond),* 109**,** 143-59.

SCHMOLL, D., WALKER, K. S., ALESSI, D. R., GREMPLER, R., BURCHELL, A., GUO, S., WALTHER, R. & UNTERMAN, T. G. 2000. Regulation of glucose-6-phosphatase gene expression by protein kinase Balpha and the forkhead transcription factor FKHR. Evidence for insulin response unit-dependent and -independent effects of insulin on promoter activity. *J Biol Chem,* 275**,** 36324-33.

SEINO, S., SEINO, M., NISHI, S. & BELL, G. I. 1989. Structure of the human insulin receptor gene and characterization of its promoter. *Proc Natl Acad Sci U S A,* 86**,** 114-8.

SENGER, D. R., CONNOLLY, D. T., VAN DE WATER, L., FEDER, J. & DVORAK, H. F. 1990. Purification and NH2-terminal amino acid sequence of guinea pig tumor-secreted vascular permeability factor. *Cancer Res,* 50**,** 1774-8.

SENGER, D. R., GALLI, S. J., DVORAK, A. M., PERRUZZI, C. A., HARVEY, V. S. & DVORAK, H. F. 1983. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science,* 219**,** 983-5.

SEOANE, J., LE, H. V., SHEN, L., ANDERSON, S. A. & MASSAGUE, J. 2004. Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. *Cell,* 117**,** 211-23.

SEOL, K. C. & KIM, S. J. 2003. Nuclear matrix association of insulin receptor and IRS-1 by insulin in osteoblast-like UMR-106 cells. *Biochem Biophys Res Commun,* 306**,** 898-904.

SHALABY, F., ROSSANT, J., YAMAGUCHI, T. P., GERTSENSTEIN, M., WU, X. F., BREITMAN, M. L. & SCHUH, A. C. 1995. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature,* 376**,** 62-6.

SHEETZ, M. J. & KING, G. L. 2002. Molecular understanding of hyperglycemia's adverse effects for diabetic complications. *Jama,* 288**,** 2579-88.

SHEN, H., CLAUSS, M., RYAN, J., SCHMIDT, A. M., TIJBURG, P., BORDEN, L., CONNOLLY, D., STERN, D. & KAO, J. 1993. Characterization of vascular permeability factor/vascular endothelial growth factor receptors on mononuclear phagocytes. *Blood,* 81**,** 2767-73.

SHEU, M. L., HO, F. M., YANG, R. S., CHAO, K. F., LIN, W. W., LIN-SHIAU, S. Y. & LIU, S. H. 2005. High glucose induces human endothelial cell apoptosis through a phosphoinositide 3-kinase-regulated cyclooxygenase-2 pathway. *Arterioscler Thromb Vasc Biol,* 25**,** 539-45.

SHIBA, T., INOGUCHI, T., SPORTSMAN, J. R., HEATH, W. F., BURSELL, S. & KING, G. L. 1993. Correlation of diacylglycerol level and protein kinase C activity in rat retina to retinal circulation. *Am J Physiol,* 265**,** E783-93.

SHIBUYA, M. & CLAESSON-WELSH, L. 2006. Signal transduction by VEGF receptors in regulation of angiogenesis and lymphangiogenesis. *Exp Cell Res,* 312**,** 549-60.

SHIBUYA, M., YAMAGUCHI, S., YAMANE, A., IKEDA, T., TOJO, A., MATSUSHIME, H. & SATO, M. 1990. Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase gene (flt) closely related to the fms family. *Oncogene,* 5**,** 519-24.

SHINKAI, A., ITO, M., ANAZAWA, H., YAMAGUCHI, S., SHITARA, K. & SHIBUYA, M. 1998. Mapping of the sites involved in ligand association and dissociation at the extracellular domain of the kinase insert domain-containing receptor for vascular endothelial growth factor. *J Biol Chem,* 273**,** 31283-8.

SHORE, V. H., WANG, T. H., WANG, C. L., TORRY, R. J., CAUDLE, M. R. & TORRY, D. S. 1997. Vascular endothelial growth factor, placenta growth factor and their receptors in isolated human trophoblast. *Placenta,* 18**,** 657-65.

SIERVO, M., RUGGIERO, D., SORICE, R., NUTILE, T., AVERSANO, M., STEPHAN, B. C. & CIULLO, M. 2010. Angiogenesis and biomarkers of cardiovascular risk in adults with metabolic syndrome. *J Intern Med,* 268**,** 338-47.

SIMS, D. E. 1986. The pericyte--a review. *Tissue Cell,* 18**,** 153-74.

SKOBE, M., ROCKWELL, P., GOLDSTEIN, N., VOSSELER, S. & FUSENIG, N. E. 1997. Halting angiogenesis suppresses carcinoma cell invasion. *Nat Med,* 3**,** 1222-7.

SKURK, C., MAATZ, H., KIM, H. S., YANG, J., ABID, M. R., AIRD, W. C. & WALSH, K. 2004. The Akt-regulated forkhead transcription factor FOXO3a controls endothelial cell viability through modulation of the caspase-8 inhibitor FLIP. *J Biol Chem,* 279**,** 1513-25.

SLEEMAN, M. W., WORTLEY, K. E., LAI, K. M., GOWEN, L. C., KINTNER, J., KLINE, W. O., GARCIA, K., STITT, T. N., YANCOPOULOS, G. D., WIEGAND, S. J. & GLASS, D. J. 2005. Absence of the lipid phosphatase SHIP2 confers resistance to dietary obesity. *Nat Med,* 11**,** 199-205.

SOMANATH, P. R., RAZORENOVA, O. V., CHEN, J. & BYZOVA, T. V. 2006. Akt1 in endothelial cell and angiogenesis. *Cell Cycle,* 5**,** 512-8.

SONG, P., WU, Y., XU, J., XIE, Z., DONG, Y., ZHANG, M. & ZOU, M. H. 2007. Reactive nitrogen species induced by hyperglycemia suppresses Akt signaling and triggers apoptosis by upregulating phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome 10) in an LKB1-dependent manner. *Circulation,* 116**,** 1585-95.

SONGYANG, Z., SHOELSON, S. E., CHAUDHURI, M., GISH, G., PAWSON, T., HASER, W. G., KING, F., ROBERTS, T., RATNOFSKY, S., LECHLEIDER, R. J. & ET AL. 1993. SH2 domains recognize specific phosphopeptide sequences. *Cell,* 72**,** 767-78.

SOWTER, H. M., CORPS, A. N., EVANS, A. L., CLARK, D. E., CHARNOCK-JONES, D. S. & SMITH, S. K. 1997. Expression and localization of the vascular endothelial growth factor family in ovarian epithelial tumors. *Lab Invest,* 77**,** 607-14.

SPIEGELMAN, B. M. & FLIER, J. S. 2001. Obesity and the regulation of energy balance. *Cell,* 104**,** 531-43.

SPIRIN, K. S., SAGHIZADEH, M., LEWIN, S. L., ZARDI, L., KENNEY, M. C. & LJUBIMOV, A. V. 1999. Basement membrane and growth factor gene expression in normal and diabetic human retinas. *Curr Eye Res,* 18**,** 490-9.

STAHL, M., DIJKERS, P. F., KOPS, G. J., LENS, S. M., COFFER, P. J., BURGERING, B. M. & MEDEMA, R. H. 2002. The forkhead transcription factor FoxO regulates transcription of p27Kip1 and Bim in response to IL-2. *J Immunol,* 168**,** 5024-31.

STAMLER, J., VACCARO, O., NEATON, J. D. & WENTWORTH, D. 1993. Diabetes, other risk factors, and 12-yr cardiovascular mortality for men screened in the Multiple Risk Factor Intervention Trial. *Diabetes Care,* 16**,** 434-44.

STANDAERT, M. L., GALLOWAY, L., KARNAM, P., BANDYOPADHYAY, G., MOSCAT, J. & FARESE, R. V. 1997. Protein kinase C-zeta as a downstream effector of phosphatidylinositol 3-kinase during insulin stimulation in rat adipocytes. Potential role in glucose transport. *J Biol Chem,* 272**,** 30075-82.

STEPHENS, L. R., HAWKINS, P. T., STANLEY, A. F., MOORE, T., POYNER, D. R., MORRIS, P. J., HANLEY, M. R., KAY, R. R. & IRVINE, R. F. 1991. myo-inositol pentakisphosphates. Structure, biological occurrence and phosphorylation to myo-inositol hexakisphosphate. *Biochem J,* 275 (Pt 2)**,** 485-99.

STEVENS, V. J., VLASSARA, H., ABATI, A. & CERAMI, A. 1977. Nonenzymatic glycosylation of hemoglobin. *J Biol Chem,* 252**,** 2998-3002.

STITT, A. W., LI, Y. M., GARDINER, T. A., BUCALA, R., ARCHER, D. B. & VLASSARA, H. 1997. Advanced glycation end products (AGEs) co-localize with AGE receptors in the retinal vasculature of diabetic and of AGE-infused rats. *Am J Pathol,* 150**,** 523-31.

STITT, A. W., MOORE, J. E., SHARKEY, J. A., MURPHY, G., SIMPSON, D. A., BUCALA, R., VLASSARA, H. & ARCHER, D. B. 1998. Advanced glycation end products in vitreous: Structural and functional implications for diabetic vitreopathy. *Invest Ophthalmol Vis Sci,* 39**,** 2517-23.

STOKOE, D., MACDONALD, S. G., CADWALLADER, K., SYMONS, M. & HANCOCK, J. F. 1994. Activation of Raf as a result of recruitment to the plasma membrane. *Science,* 264**,** 1463-7.

SUKHANOV, S., HIGASHI, Y., SHAI, S. Y., VAUGHN, C., MOHLER, J., LI, Y., SONG, Y. H., TITTERINGTON, J. & DELAFONTAINE, P. 2007. IGF-1 reduces inflammatory responses, suppresses oxidative stress, and decreases atherosclerosis progression in ApoE-deficient mice. *Arterioscler Thromb Vasc Biol,* 27**,** 2684-90.

SUN, X. J., ROTHENBERG, P., KAHN, C. R., BACKER, J. M., ARAKI, E., WILDEN, P. A., CAHILL, D. A., GOLDSTEIN, B. J. & WHITE, M. F. 1991. Structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. *Nature,* 352**,** 73-7.

SUSARLA, R., WATKINSON, J. C. & EGGO, M. C. 2007. Regulation of plasminogen activators in human thyroid follicular cells and their relationship to differentiated function. *J Cell Physiol,* 212**,** 643-54.

SZEKANECZ, Z. & KOCH, A. E. 2007. Mechanisms of Disease: angiogenesis in inflammatory diseases. *Nat Clin Pract Rheumatol,* 3**,** 635-43.

TAKAHASHI, T., SHIRASAWA, T., MIYAKE, K., YAHAGI, Y., MARUYAMA, N., KASAHARA, N., KAWAMURA, T., MATSUMURA, O., MITARAI, T. & SAKAI, O. 1995. Protein tyrosine kinases expressed in glomeruli and cultured glomerular cells: Flt-1 and VEGF expression in renal mesangial cells. *Biochem Biophys Res Commun,* 209**,** 218-26.

TAKAHASHI, T., UENO, H. & SHIBUYA, M. 1999. VEGF activates protein kinase C-dependent, but Ras-independent Raf-MEK-MAP kinase pathway for DNA synthesis in primary endothelial cells. *Oncogene,* 18**,** 2221-30.

TAKAISHI, H., KONISHI, H., MATSUZAKI, H., ONO, Y., SHIRAI, Y., SAITO, N., KITAMURA, T., OGAWA, W., KASUGA, M., KIKKAWA, U. & NISHIZUKA, Y. 1999. Regulation of nuclear translocation of forkhead transcription factor AFX by protein kinase B. *Proc Natl Acad Sci U S A,* 96**,** 11836-41.

TAKEI, Y., KADOMATSU, K., YUZAWA, Y., MATSUO, S. & MURAMATSU, T. 2004. A small interfering RNA targeting vascular endothelial growth factor as cancer therapeutics. *Cancer Res,* 64**,** 3365-70.

TAMEMOTO, H., KADOWAKI, T., TOBE, K., YAGI, T., SAKURA, H., HAYAKAWA, T., TERAUCHI, Y., UEKI, K., KABURAGI, Y., SATOH, S. & ET AL. 1994. Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. *Nature,* 372**,** 182-6.

TANAKA, J., QIANG, L., BANKS, A. S., WELCH, C. L., MATSUMOTO, M., KITAMURA, T., IDO-KITAMURA, Y., DEPINHO, R. A. & ACCILI, D. 2009. Foxo1 links hyperglycemia to LDL oxidation and endothelial nitric oxide synthase dysfunction in vascular endothelial cells. *Diabetes,* 58**,** 2344-54.

TANG, X., POWELKA, A. M., SORIANO, N. A., CZECH, M. P. & GUILHERME, A. 2005. PTEN, but not SHIP2, suppresses insulin signaling through the phosphatidylinositol 3-kinase/Akt pathway in 3T3-L1 adipocytes. *J Biol Chem,* 280**,** 22523-9.

TANIGUCHI, C. M., UEKI, K. & KAHN, R. 2005. Complementary roles of IRS-1 and IRS-2 in the hepatic regulation of metabolism. *J Clin Invest,* 115**,** 718-27.

TERMAN, B. I., CARRION, M. E., KOVACS, E., RASMUSSEN, B. A., EDDY, R. L. & SHOWS, T. B. 1991. Identification of a new endothelial cell growth factor receptor tyrosine kinase. *Oncogene,* 6**,** 1677-83.

TERMAN, B. I., JANI-SAIT, S., CARRION, M. E. & SHOWS, T. B. 1992. The KDR gene maps to human chromosome 4q31.2----q32, a locus which is distinct from locations for other type III growth factor receptor tyrosine kinases. *Cytogenet Cell Genet,* 60**,** 214-5.

THIEME, H., AIELLO, L. P., TAKAGI, H., FERRARA, N. & KING, G. L. 1995. Comparative analysis of vascular endothelial growth factor receptors on retinal and aortic vascular endothelial cells. *Diabetes,* 44**,** 98-103.

THOMAS, J. H. 1993. Chemosensory regulation of development in C. elegans. *Bioessays,* 15**,** 791-7.

TISCHER, E., MITCHELL, R., HARTMAN, T., SILVA, M., GOSPODAROWICZ, D., FIDDES, J. C. & ABRAHAM, J. A. 1991. The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. *J Biol Chem,* 266**,** 11947-54.

TOTHOVA, Z., KOLLIPARA, R., HUNTLY, B. J., LEE, B. H., CASTRILLON, D. H., CULLEN, D. E., MCDOWELL, E. P., LAZO-KALLANIAN, S., WILLIAMS, I. R., SEARS, C., ARMSTRONG, S. A., PASSEGUE, E., DEPINHO, R. A. & GILLILAND, D. G. 2007. FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. *Cell,* 128**,** 325-39.

TSCHOPP, O., YANG, Z. Z., BRODBECK, D., DUMMLER, B. A., HEMMINGS-MIESZCZAK, M., WATANABE, T., MICHAELIS, T., FRAHM, J. & HEMMINGS, B. A. 2005. Essential role of protein kinase B gamma (PKB gamma/Akt3) in postnatal brain development but not in glucose homeostasis. *Development,* 132**,** 2943-54.

TSENG, Y. H., KRIAUCIUNAS, K. M., KOKKOTOU, E. & KAHN, C. R. 2004. Differential roles of insulin receptor substrates in brown adipocyte differentiation. *Mol Cell Biol,* 24**,** 1918-29.

TURECKOVA, J., WILSON, E. M., CAPPALONGA, J. L. & ROTWEIN, P. 2001. Insulin-like growth factor-mediated muscle differentiation: collaboration between phosphatidylinositol 3-kinase-Akt-signaling pathways and myogenin. *J Biol Chem,* 276**,** 39264-70.

UEKI, K., KONDO, T. & KAHN, C. R. 2004. Suppressor of cytokine signaling 1 (SOCS-1) and SOCS-3 cause insulin resistance through inhibition of tyrosine phosphorylation of insulin receptor substrate proteins by discrete mechanisms. *Mol Cell Biol,* 24**,** 5434-46.

UI, M., OKADA, T., HAZEKI, K. & HAZEKI, O. 1995. Wortmannin as a unique probe for an intracellular signalling protein, phosphoinositide 3-kinase. *Trends Biochem Sci,* 20**,** 303-7.

VAISMAN, N., GOSPODAROWICZ, D. & NEUFELD, G. 1990. Characterization of the receptors for vascular endothelial growth factor. *J Biol Chem,* 265**,** 19461-6.

VAN DE VEIRE, N. R., DELGADO, V., SCHUIJF, J. D., VAN DER WALL, E. E., SCHALIJ, M. J. & BAX, J. J. 2009. The role of non-invasive imaging in patient selection. *Europace,* 11 Suppl 5**,** v32-9.

VAN DE VEIRE, S., STALMANS, I., HEINDRYCKX, F., OURA, H., TIJERAS-RABALLAND, A., SCHMIDT, T., LOGES, S., ALBRECHT, I., JONCKX, B., VINCKIER, S., VAN STEENKISTE, C., TUGUES, S., ROLNY, C., DE MOL, M., DETTORI, D., HAINAUD, P., COENEGRACHTS, L., CONTRERES, J. O., VAN BERGEN, T., CUERVO, H., XIAO, W. H., LE HENAFF, C., BUYSSCHAERT, I., KHARABI MASOULEH, B., GEERTS, A., SCHOMBER, T., BONNIN, P., LAMBERT, V., HAUSTRAETE, J., ZACCHIGNA, S., RAKIC, J. M., JIMENEZ, W., NOEL, A., GIACCA, M., COLLE, I., FOIDART, J. M., TOBELEM, G., MORALES-RUIZ, M., VILAR, J., MAXWELL, P., VINORES, S. A., CARMELIET, G., DEWERCHIN, M., CLAESSON-WELSH, L., DUPUY, E., VAN VLIERBERGHE, H., CHRISTOFORI, G., MAZZONE, M., DETMAR, M., COLLEN, D. & CARMELIET, P. 2010. Further pharmacological and genetic evidence for the efficacy of PlGF inhibition in cancer and eye disease. *Cell,* 141**,** 178-90.

VEVES, A., AKBARI, C. M., PRIMAVERA, J., DONAGHUE, V. M., ZACHAROULIS, D., CHRZAN, J. S., DEGIROLAMI, U., LOGERFO, F. W. & FREEMAN, R. 1998. Endothelial dysfunction and the expression of endothelial nitric oxide synthetase in diabetic neuropathy, vascular disease, and foot ulceration. *Diabetes,* 47**,** 457-63.

VIDAL, F., ARAGONES, J., ALFRANCA, A. & DE LANDAZURI, M. O. 2000. Up-regulation of vascular endothelial growth factor receptor Flt-1 after endothelial denudation: role of transcription factor Egr-1. *Blood,* 95**,** 3387-95.

VIGLIETTO, G., MAGLIONE, D., RAMBALDI, M., CERUTTI, J., ROMANO, A., TRAPASSO, F., FEDELE, M., IPPOLITO, P., CHIAPPETTA, G., BOTTI, G. & ET AL. 1995. Upregulation of vascular endothelial growth factor (VEGF) and downregulation of placenta growth factor (PlGF) associated with malignancy in human thyroid tumors and cell lines. *Oncogene,* 11**,** 1569-79.

VLAHOS, C. J., MATTER, W. F., HUI, K. Y. & BROWN, R. F. 1994. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J Biol Chem,* 269**,** 5241-8.

VOROS, G., MAQUOI, E., DEMEULEMEESTER, D., CLERX, N., COLLEN, D. & LIJNEN, H. R. 2005. Modulation of angiogenesis during adipose tissue development in murine models of obesity. *Endocrinology,* 146**,** 4545-54.

VUORELA, P., HATVA, E., LYMBOUSSAKI, A., KAIPAINEN, A., JOUKOV, V., PERSICO, M. G., ALITALO, K. & HALMESMAKI, E. 1997. Expression of vascular endothelial growth factor and placenta growth factor in human placenta. *Biol Reprod,* 56**,** 489-94.

WADA, T., SASAOKA, T., ISHIKI, M., HORI, H., HARUTA, T., ISHIHARA, H. & KOBAYASHI, M. 1999. Role of the Src homology 2 (SH2) domain and C-terminus tyrosine phosphorylation sites of SH2-containing inositol phosphatase (SHIP) in the regulation of insulin-induced mitogenesis. *Endocrinology,* 140**,** 4585-94.

WALTENBERGER, J., CLAESSON-WELSH, L., SIEGBAHN, A., SHIBUYA, M. & HELDIN, C. H. 1994. Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. *J Biol Chem,* 269**,** 26988-95.

WANG, G. L. & SEMENZA, G. L. 1995. Purification and characterization of hypoxia-inducible factor 1. *J Biol Chem,* 270**,** 1230-7.

WARREN, R. S., YUAN, H., MATLI, M. R., FERRARA, N. & DONNER, D. B. 1996. Induction of vascular endothelial growth factor by insulin-like growth factor 1 in colorectal carcinoma. *J Biol Chem,* 271**,** 29483-8.

WEI, S. C., TSAO, P. N., YU, S. C., SHUN, C. T., TSAI-WU, J. J., WU, C. H., SU, Y. N., HSIEH, F. J. & WONG, J. M. 2005. Placenta growth factor expression is correlated with survival of patients with colorectal cancer. *Gut,* 54**,** 666-72.

WEIGEL, D., JURGENS, G., KUTTNER, F., SEIFERT, E. & JACKLE, H. 1989. The homeotic gene fork head encodes a nuclear protein and is expressed in the terminal regions of the Drosophila embryo. *Cell,* 57**,** 645-58.

WEISBERG, S. P., HUNTER, D., HUBER, R., LEMIEUX, J., SLAYMAKER, S., VADDI, K., CHARO, I., LEIBEL, R. L. & FERRANTE, A. W., JR. 2006. CCR2 modulates inflammatory and metabolic effects of high-fat feeding. *J Clin Invest,* 116**,** 115-24.

WESTMAN, S. 1968. Development of the obese-hyperglycaemic syndrome in mice. *Diabetologia,* 4**,** 141-9.

WILLETT, C. G., BOUCHER, Y., DI TOMASO, E., DUDA, D. G., MUNN, L. L., TONG, R. T., CHUNG, D. C., SAHANI, D. V., KALVA, S. P., KOZIN, S. V., MINO, M., COHEN, K. S., SCADDEN, D. T., HARTFORD, A. C., FISCHMAN, A. J., CLARK, J. W., RYAN, D. P., ZHU, A. X., BLASZKOWSKY, L. S., CHEN, H. X., SHELLITO, P. C., LAUWERS, G. Y. & JAIN, R. K. 2004. Direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer. *Nat Med,* 10**,** 145-7.

WITHERS, D. J., GUTIERREZ, J. S., TOWERY, H., BURKS, D. J., REN, J. M., PREVIS, S., ZHANG, Y., BERNAL, D., PONS, S., SHULMAN, G. I., BONNER-WEIR, S. & WHITE, M. F. 1998. Disruption of IRS-2 causes type 2 diabetes in mice. *Nature,* 391**,** 900-4.

WOODS, Y. L., COHEN, P., BECKER, W., JAKES, R., GOEDERT, M., WANG, X. & PROUD, C. G. 2001a. The kinase DYRK phosphorylates protein-synthesis initiation factor eIF2Bepsilon at Ser539 and the microtubule-associated protein tau at Thr212: potential role for DYRK as a glycogen synthase kinase 3-priming kinase. *Biochem J,* 355**,** 609-15.

WOODS, Y. L., RENA, G., MORRICE, N., BARTHEL, A., BECKER, W., GUO, S., UNTERMAN, T. G. & COHEN, P. 2001b. The kinase DYRK1A phosphorylates the transcription factor FKHR at Ser329 in vitro, a novel in vivo phosphorylation site. *Biochem J,* 355**,** 597-607.

XU, J. & LIAO, K. 2004. Protein kinase B/AKT 1 plays a pivotal role in insulin-like growth factor-1 receptor signaling induced 3T3-L1 adipocyte differentiation. *J Biol Chem,* 279**,** 35914-22.

XU, L., COCHRAN, D. M., TONG, R. T., WINKLER, F., KASHIWAGI, S., JAIN, R. K. & FUKUMURA, D. 2006. Placenta growth factor overexpression inhibits tumor growth, angiogenesis, and metastasis by depleting vascular endothelial growth factor homodimers in orthotopic mouse models. *Cancer Res,* 66**,** 3971-7.

XUAN, Z. & ZHANG, M. Q. 2005. From worm to human: bioinformatics approaches to identify FOXO target genes. *Mech Ageing Dev,* 126**,** 209-15.

YANCOPOULOS, G. D., DAVIS, S., GALE, N. W., RUDGE, J. S., WIEGAND, S. J. & HOLASH, J. 2000. Vascular-specific growth factors and blood vessel formation. *Nature,* 407**,** 242-8.

YANG, J. Y., ZONG, C. S., XIA, W., YAMAGUCHI, H., DING, Q., XIE, X., LANG, J. Y., LAI, C. C., CHANG, C. J., HUANG, W. C., HUANG, H., KUO, H. P., LEE, D. F., LI, L. Y., LIEN, H. C., CHENG, X., CHANG, K. J., HSIAO, C. D., TSAI, F. J., TSAI, C. H., SAHIN, A. A., MULLER, W. J., MILLS, G. B., YU, D., HORTOBAGYI, G. N. & HUNG, M. C. 2008. ERK promotes tumorigenesis by inhibiting FOXO3a via MDM2-mediated degradation. *Nat Cell Biol,* 10**,** 138-48.

YANG, W., AHN, H., HINRICHS, M., TORRY, R. J. & TORRY, D. S. 2003. Evidence of a novel isoform of placenta growth factor (PlGF-4) expressed in human trophoblast and endothelial cells. *J Reprod Immunol,* 60**,** 53-60.

YANG, X. & CEPKO, C. L. 1996. Flk-1, a receptor for vascular endothelial growth factor (VEGF), is expressed by retinal progenitor cells. *J Neurosci,* 16**,** 6089-99.

YAO, Y. G., YANG, H. S., CAO, Z., DANIELSSON, J. & DUH, E. J. 2005. Upregulation of placental growth factor by vascular endothelial growth factor via a post-transcriptional mechanism. *FEBS Lett,* 579**,** 1227-34.

YEAGLEY, D., GUO, S., UNTERMAN, T. & QUINN, P. G. 2001. Gene- and activation-specific mechanisms for insulin inhibition of basal and glucocorticoid-induced insulin-like growth factor binding protein-1 and phosphoenolpyruvate carboxykinase transcription. Roles of forkhead and insulin response sequences. *J Biol Chem,* 276**,** 33705-10.

YOAKIM, M., HOU, W., SONGYANG, Z., LIU, Y., CANTLEY, L. & SCHAFFHAUSEN, B. 1994. Genetic analysis of a phosphatidylinositol 3-kinase SH2 domain reveals determinants of specificity. *Mol Cell Biol,* 14**,** 5929-38.

YONEKURA, H., SAKURAI, S., LIU, X., MIGITA, H., WANG, H., YAMAGISHI, S., NOMURA, M., ABEDIN, M. J., UNOKI, H., YAMAMOTO, Y. & YAMAMOTO, H. 1999. 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**,** 35172-8.

YOON, J. C., PUIGSERVER, P., CHEN, G., DONOVAN, J., WU, Z., RHEE, J., ADELMANT, G., STAFFORD, J., KAHN, C. R., GRANNER, D. K., NEWGARD, C. B. & SPIEGELMAN, B. M. 2001. Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1. *Nature,* 413**,** 131-8.

YUN, S. J., KIM, E. K., TUCKER, D. F., KIM, C. D., BIRNBAUM, M. J. & BAE, S. S. 2008. Isoform-specific regulation of adipocyte differentiation by Akt/protein kinase Balpha. *Biochem Biophys Res Commun,* 371**,** 138-43.

ZENG, G., NYSTROM, F. H., RAVICHANDRAN, L. V., CONG, L. N., KIRBY, M., MOSTOWSKI, H. & QUON, M. J. 2000. Roles for insulin receptor, PI3-kinase, and Akt in insulin-signaling pathways related to production of nitric oxide in human vascular endothelial cells. *Circulation,* 101**,** 1539-45.

ZENG, G. & QUON, M. J. 1996. Insulin-stimulated production of nitric oxide is inhibited by wortmannin. Direct measurement in vascular endothelial cells. *J Clin Invest,* 98**,** 894-8.

ZHANG, H., PALMER, R., GAO, X., KREIDBERG, J., GERALD, W., HSIAO, L., JENSEN, R. V., GULLANS, S. R. & HABER, D. A. 2003. Transcriptional activation of placental growth factor by the forkhead/winged helix transcription factor FoxD1. *Curr Biol,* 13**,** 1625-9.

ZHANG, L., CHEN, J., KE, Y., MANSEL, R. E. & JIANG, W. G. 2005. Expression of Placenta growth factor (PlGF) in non-small cell lung cancer (NSCLC) and the clinical and prognostic significance. *World J Surg Oncol,* 3**,** 68.

ZHANG, W., PATIL, S., CHAUHAN, B., GUO, S., POWELL, D. R., LE, J., KLOTSAS, A., MATIKA, R., XIAO, X., FRANKS, R., HEIDENREICH, K. A., SAJAN, M. P., FARESE, R. V., STOLZ, D. B., TSO, P., KOO, S. H., MONTMINY, M. & UNTERMAN, T. G. 2006. FoxO1 regulates multiple metabolic pathways in the liver: effects on gluconeogenic, glycolytic, and lipogenic gene expression. *J Biol Chem,* 281**,** 10105-17.

ZHANG, Y., PROENCA, R., MAFFEI, M., BARONE, M., LEOPOLD, L. & FRIEDMAN, J. M. 1994. Positional cloning of the mouse obese gene and its human homologue. *Nature,* 372**,** 425-32.

ZHAO, B., CAI, J. & BOULTON, M. 2004. Expression of placenta growth factor is regulated by both VEGF and hyperglycaemia via VEGFR-2. *Microvasc Res,* 68**,** 239-46.

ZHOU, J., SCHMID, T., SCHNITZER, S. & BRUNE, B. 2006. Tumor hypoxia and cancer progression. *Cancer Lett,* 237**,** 10-21.

ZICHE, M., MAGLIONE, D., RIBATTI, D., MORBIDELLI, L., LAGO, C. T., BATTISTI, M., PAOLETTI, I., BARRA, A., TUCCI, M., PARISE, G., VINCENTI, V., GRANGER, H. J., VIGLIETTO, G. & PERSICO, M. G. 1997. Placenta growth factor-1 is chemotactic, mitogenic, and angiogenic. *Lab Invest,* 76**,** 517-31.

APPENDIX I – Chemical Reagents and Suppliers

Ammonium acetate	Sigma, Poole, UK
40% Acrylamide, 2% bis acrylamide	Bio-Rad, UK
Agarose (molecular grade)	Sigma, Poole, UK
Ammonium persulphate	Sigma, Poole, UK
BCA protein assay	Bio-Rad, Hertfordshire, UK
Bovine serum albumin	Sigma, Poole, UK
Bromophenol blue	Sigma, Poole, UK
Promega cDNA synthesis kit	Promega, Madison, USA
Caesium chloride	Sigma, Poole, UK
Collagenase type V	Sigma, Pool, UK
ChIP kit (17-371)	Millipore, Watford, UK
Chloroform	Sigma, Poole, UK
DAB	DAKO, Cambrodgeshire, UK
DMSO	Sigma, Poole, UK
DTT	Pharmacia, Herts, UK
ECL detection kit	Amersham, Buckinghamshire, UK
EDTA	Sigma, Poole, UK
Epidermal growth factor	ReliaTECH, Braunschweig, Germany
ELISA substrate	R&D systems, Abington, UK
Endothelial cell basal medium (EBM)	Lonza, Slough, UK
Ethanol (99.7-100 %):	BDH, Poole, UK
Ethidium bromide	Sigma, Poole, UK
Ex-GEN-500	Fermentas,York, UK

Foetal bovine calf serum (F7524)	Gibco, Paisley, Scotland
FOXO1 plasmid	Cambridge, MA, USA
FOXO3a plasmid	Cambridge, MA, USA
Gelatin	Sigma, Poole, UK
Promega Gene reporter assay kit	Promega, Madison, USA
Glycogen	Sigma, Poole, UK
Glycerol	Sigma, Poole, UK
Glycine	Sigma, Poole, UK
HAEC	Lonza,Slough, UK
Heparin	Sigma, Poole, UK
HBSS	Sigma, Poole, UK
HEK 293 Amaxa kit V	Lonza,Slough, UK
HEPES	Sigma, Poole, UK
HUVEC Amaxa Kit I	Lonza,Slough, UK
Hydrogen peroxide:	Sigma, Poole, UK
IGF-1	ReliaTECH, Braunschweig, Germany
Isohelenin	Calbiochem, Nottingham, UK
Isopropanol	Sigma, Poole, UK
Kaleidoscope prestained standards:	Bio-Rad, Hertfordshire, UK
Kodak, X-Omat AR film	Sigma, Poole, UK
L-Glutamine	Sigma, Poole, UK
Leupeptin	Sigma, Poole, UK
LY294002	Calbiochem, Nottingham, UK
Marvel dried milk	Sainsbury's, Birmingham, UK

M 199 medium	ICN, Basingstoke, UK
MCDB 131	Invitrogen, Paisley, UK)
Methanol	Sigma, Poole, UK
PCR kit	Bioline, London, UK
PCR loading dye	Sigma, Poole, UK
Penicillin	Gibco, Paisley, Scotland
Pepstatin A	Sigma, Poole, UK
Phenol:chloroform	Sigma, Poole, UK
Phenylmethylsulfonyl fluoride (PMSF)	Sigma, Poole, UK
Phosphate buffered saline tablets (PBS)	Lonza, Slough, UK
Phosphatase inhibitors	Sigma, Poole, UK
PlGF duoset ELISA (human and mouse)	R&D systems, Abington, UK
PlGF:VEGF antibody (892984)	R&D systems, Abington, UK
PlGF:VEGF Recombinant (892985)	R&D systems, Abington, UK
Primers	Eurogentec, Southampton, UK
Protease inhibitors	Sigma, Poole, UK
qPCR kit	Quantace, London, UK
RPMI 1640	Gibco, Paisley, Scotland
RIPA buffer	Bio-Rad,Hertfordshire, UK
RNA extraction kit	Norgen, Staffordshire, UK
siRNA	Dharmacon, Loughborough, UK
Sodium dodecyl sulphate:	Sigma, Poole, UK
Streptomycin	Gibco, Paisley, Scotland
TEMED	Bio-Rad, Hertfordshire, UK

Tris	Sigma, Poole, UK
Trypsin (T3924)	Sigma, Poole, UK
Tween-20	Sigma, Poole, UK
VEGF-A	ReliaTECH, Braunschweig, Germany
VEGF-A duoset ELISA	R&D systems, Abington, UK
VEGFR-2 (**841245**)	R&D systems, Abington, UK
VEGFR-2 antibody (**841243**)	R&D systems, Abington, UK
VEGFR-2 biotinylated antibody (**841244**)	R&D systems, Abington, UK

All other cell culture reagents and chemicals were obtained from Sigma, Poole, UK.

APPENDIX II – Antibodies and Suppliers

Antibody	Species raised in	Dilution for WB	Code	Source
β-actin	mouse	1:20,000	A-1978	Cell Signaling Tech, Herts, UK
Akt	rabbit	1:1000	9272	Cell Signaling Tech, Herts, UK
Akt (phospho ser-473)	rabbit	1:500	9271	Cell Signaling Tech, Herts, UK
PTEN	mouse	1:200	9552	NEB, Ipswich, UK
VEGFR-1 (flt-11)	mouse	1:100	V4262	Sigma, Poole, UK
Anti-HA	mouse	1:1000	11583816001	Roche, Hertfordshire, UK
FOXO1 (Phospho ser256)	rabbit	1:1000	9461	Cell Signaling Tech, Herts, UK
FOXO1	rabbit	1:1000	9462	Cell Signaling Tech, Herts, UK
Hexon	rabbit	1:1000	ab8249	Abcam,Cambridge, UK

SECONDARY ANTIBODY CONJUGATES

HRP IgG conjugates obtained from Vector Labs and used at 1:5000 for WB.

APPENDIX III – Equipment and Suppliers

Amaxa electroporator	Lonza, Slough, UK
Bench top microcentrifuge:	Phillip Harris, Staffs, UK
Cell culture pipettes:	Fahrenheit Lab Supplies, UK
Centrifuge (Sigma 2K 15)	Sigma, Poole, UK
Centrifuge (80 K ultrcentrifuge)	Beckman Coulter, High Wycombe, UK
Class II cell culture cabinets:	Triple Red, Oxfordshire, UK
Conical tubes (15 ml):	Gibco BRL, Paisley, UK
Coverglass:	Surgipath, Staffs, UK
Cryovials:	Gibco BRL, Paisley, UK
Diagenode Bioruptor Sonicator	Wolf laboratories Ltd, York, UK
Disposable Scalpels:	Appleton Woods, Birmingham, UK
microfuge tubes	Starstedt, Leicester UK
Falcon tubes (15 and 50 ml):	Falcon/BDH, Poole, UK
Filters (0.22 mm):	Millipore, Herts, UK
Filter units (swinnex 47 and 22):	Millipore, Herts, UK
Flasks (25 and 75 cm):	Falcon/BDH, Poole, UK
Gilson pipettes:	Anachem, Buckinghamshire, UK
Gilson tips (blue):	Starstedt, Leicester, UK
Gilson tips (yellow):	Starstedt, Leicester, UK

Glass Pasteur pipettes 9':	Fisher Scientific, Loughborough, UK
Glassware:	Phillip Harris, Staffs, UK
Haemocytometer	Hawksley, Sussex, UK
Heparin-Sepharose column (HiTrap):	Amersham Bioscience, UK
Hybond ECL nitrocellulose membrane :	Amersham, Buckinghamshire, UK
Hybridisation oven/shaker (SI 20H):	Stuart Scientific, UK
Horizontal gel electrophoresis system:	Gibco, Paisley, Scotland
Luminometer	Berthold, Staffordshire, UK
Microscope (Nikon, TS-100)	Nikon, Surry, UK
Mini sub DNA gel:	Bio-Rad, Hemel Hempstead, UK
Mr Frosty	Nalgene, Rochester, NY, USA
Multiwell Plates (12 and 24-wells):	Falcon/BDH, Poole, UK
Omnigene thermal cycler:	Hybaid, Middlesex, UK
PCR thermocycler	Sunquest, Arizona, USA
pH meter:	Corning costar, High Wycombe, UK
Pipettes (5ml and 10 ml):	Falcon/BDH, Poole, UK
Precellys Tissue Homogeniser	VWR, Leicestershire, UK
Polytron Homogeniser PT1200:	Phillip Harris, Staffs, UK
Rotary shaker (R100):	Appleton Woods, Birminghamn, UK
Rotor-gene 6000	Corbett, NSW, Australia

Shaking orbital incubator:	Appleton Woods, Birminghamn, UK
Shaking water bath:	Grant Instruments, Cambridge, UK
Spectrophotometer	Amersham Biosciences, Amersham, UK
Syringes (1ml - 50 ml):	Appleton Woods, Birmingham, UK
Transfer-blot electrophoresis cell:	Bio-Rad, Hemel Hempstead, UK
Ultracentrifuge:	Phillip Harris, Staffs, UK
Universals (30 ml):	Phillip Harris, Poole, UK
U.V light system:	Appleton Woods, Birmingham, UK
Vertical gel electrophoresis unit:	Bio-Rad, Hemel Hempstead, UK
Water-Jacketed Incubator:	Sanyo-Gallenkamp, Leicester, UK
Weight Balance:	Sartorius Limited, Surrey, UK
Whatman 3 MM paper:	Whatman, Kent, UK
Xograph developer	Geneflow, Staffordshire, UK

APPENDIX IV – Solutions and Buffers

PBS (10X) (pH7.3)	1.440 g/L KH_2PO_4 9.0000 g/L NaCl 7.950 g/L Na_2HPO_4
TBS (pH 7.6)	20 mM Tris 73.1 mM NaCl
TBS-T (pH7.6)	20 mM Tris 73.1 mM NaCl 0.1% (v/v) Tween
SDS-PAGE Running Buffer (pH 8.3)	50 mM Tris 384 mM Glycine 3.47 mM SDS
Gel to Membrane Transfer Buffer (pH 8.3)	39 mM Glycine 48 mM Tris 1.3 mM SDS 20 % (v/v) Methanol
Blocking Buffer	TBS pH 7.6 8% (w/v) Fat-free milk
Antibody Diluent	TBS pH 7.6 0.5% (w/v) BSA
Reducing Sample Buffer	125 mM Tris pH 6.8 20% (v/v) Glycerol 4% (w/v) SDS 200 mM DTT

	1:10,000 (w/v) Bromophenol Blue
Non - Reducing Sample Buffer	125 mM Tris pH 6.8 20% (v/v) Glycerol 4% (w/v) SDS 1:10,000 (w/v) Bromophenol Blue
Stripping Buffer	0.2 M Na OH
RIPA Cell Lysis Buffer	50 mM Tris pH 7.4 1 % IGEPAL 0.25 % Sodium deoxycholate 150 mM NaCl 1 mM EGTA 1 mM NaF, Na_3VO_4 and PMSF 1 µg/ml Apoprotin, Leupeptin and Pepstatin (code: 89900 thermo scientific, Rockford, USA)

GEL RECIPES

Running gel (1.5 mm spacer)

For 1 mini-gel	6 %	8 %	10 %	12 %	15 %
dH_2O (ml)	5.45	4.78	4.12	3.45	2.45
1.5 M Tris pH 8.8 (ml)	2.5	2.5	2.5	2.5	2.5
Acrylamide (ml)	2	2.67	3.33	4	5
10 % APS (µl)	50	50	50	50	50
TEMED (µl)	5	5	5	5	5
Total (ml)	10	10	10	10	10

Stacking gel (1.5 mm spacer)

For 1 mini-gel	1x
dH_2O (ml)	3.07
1.5 M Tris pH 8.8 (ml)	1.25
Acrylamide (ml)	0.65
10 % APS (µl)	25
TEMED (µl)	5
Total (ml)	5

ELISA reagent diluents	1% BSA in PBS, pH 7.2 - 7.4
ELISA washing buffer	0.05% Tween 20 in PBS, pH 7.2 - 7.4
ELISA Substrate Solution	1:1 mixture of Colour Reagent A (H_2O_2) And Colour Reagent B (Tetramethylbenzidine)
ELISA stop solution	2 N H_2SO_4

Dialysis buffer	(5liters)(pH 8.0)	10 mM Tris base
		2 mM $MgCl_2$
		4% (w/v) sucrose

Tris-Borate-EDTA (pH 8.0)	89 mM Tris Base
	89 mM Boric Acid
	20 mM EDTA

8. Published Articles Attached

[Not available in the digital version of this thesis]